Mammalian expression and purification system using *Strep*-tag and/or 6xHistidine-tag

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The Ni-NTA resin is manufactured by QIAGEN. Hoffmann-La Roche owns patents and patent applications pertaining to the application of Ni-NTA resin (U.S. patent 4.877.830, U.S. patent 5.047.513, EP 253 303 B1) and to the method of purifying 6xHistidine-tagged proteins using 6xHistidine-tag coding vectors (U.S. patent 5.284.933, U.S. patent 5.130.663, EP 282 042 B1). All purification of 6xHistidine-tagged proteins by metal affinity chromatography for commercial purposes, and the commercial use of proteins so purified, require a license from Hoffmann-La Roche. Further information about licenses for commercial use is available from QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden, Germany.

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Content

1 Introduction

1.1 pEXPR-IBA vectors

pEXPR-IBA vectors are designed for high-level expression of recombinant proteins in mammalian hosts and for their subsequent purification via *Strep*-tag and/or 6xHistidine-tag. The human cytomegalovirus immediate-early (CMV) promoter provides strong expression in a wide range of mammalian cells. To prolong expression in transfected cells, the vector will replicate in cell lines that are latently infected with SV40 large T antigen (e.g. COS7). In addition, Neomycin resistance gene allows direct selection of stable cell lines. pEXPR-IBA42 and pEXPR-IBA44 are designed for secretion of the recombinant protein into the medium. pEXPR-IBA vectors are available in a multitude of different versions and MCS are compatible with the corresponding pASK-IBA or pPR-IBA vectors for bacterial expression (e.g. pEXPR-IBA3 ≅ pASK-IBA3; pEXPR-IBA5 ≅ pASK-IBA5 etc.).

Name	BM40 signal peptide	N-terminal tag	Cleavage	C-terminal tag
pEXPR-IBA3	no	no	no	Strep-tag
pEXPR-IBA5	no	Strep-tag	no	no
pEXPR-IBA7	no	Strep-tag	Factor Xa	no
pEXPR-IBA13	no	Strep-tag	Thrombin	no
pEXPR-IBA15	no	Strep-tag	Enterokinase	no
pEXPR-IBA42	yes	6xHistidine-tag	no	Strep-tag
pEXPR-IBA44	yes	Strep-tag	no	6xHistidine-tag

1.2 *Strep*-tag®/*Strep*-Tactin® system

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].
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 (2.5 mM) desthiobiotin. The *Strep*-tag/Strep-Tactin 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 (25 - 100 nmol/ml) depends on the *Strep*-Tactin matrices and on the fused recombinant protein.
Because of its small size, *Strep*-tag 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.iba-go.com.

Reagent	Concentration
Reducing Agents	
DTT	50 mM
β-mercaptoethanol	50 mM
Non-Ionic Detergents	
C_8E_4 Octyltetraoxyethylene	0.88 %
$C_{10}E_5$; Decylpentaoxyethylene	0.12 %
$C_{10}E_6$	0.03 %
$C_{12}E_8$	0.005 %
$C_{12}E_9$; Dodecyl nonaoxyethylene (Thesit)	0.023 %
DM; Decyl-ß-D-maltoside	0.35 %
LM; N-dodecyl-β-D-maltoside	0.007 %
NG; N-nonyl-β-D-glucopyranoside	0.2 %
OG; N-octyl-β-D-glucopyranoside	2.34 %
TX; Triton X-100	2 %
Tween 20	2 %
Ionic Detergents	
N-lauryl-sarcosine	2 %
8-HESO;N-octyl-2-hydroxy-ethylsulfoxide	1,32 %
SDS; Sodium-N-dodecyl sulfate	0.1 %
Zwitter-Ionic Detergents	
CHAPS	0.1 %
DDAO; N-decyl-N,N-dimethylamine-N-oxide	0.034 %
LDAO; N-dodecyl-N,N-dimethylamine-N-oxide	0.13 %
Others	
Ammonium sulfate $(NH_4)_2SO_4$	2 M
$CaCl_2$	1 M
EDTA	50 mM
Ethanol	10 %
Guanidine	1 M
Glycerol	25 %
Imidazole	250 mM
$MgCl_2$	1 M
NaCl	5 M
Urea	1 M

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

*** Note:These reagents have been successfully tested for the purification of e.g. GAPDH-*Strep*-tag with concentrations up to those mentioned. For most reagents higher concentrations may be possible, though. However, since binding depends on the sterical accessibility of *Strep*-tag in the context of the particular protein the maximal concentration may be different for certain proteins.**

1.3 6xHistidine-tag in combination with the *Strep*-tag

The 6xHistidine-tag Ni-NTA interaction is based on the selectivity and high affinity of Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues [4,5]. In addition to the *Strep*-tag, we have included the 6xHistidine-tag in some vectors creating double tag proteins. The 6xHistidine-tag serves for concentrating the recombinant protein and for removing biotin, which may be present in the medium. Since biotin binds to *Strep*-Tactin in competition to the *Strep*-tag, biotin in the medium would hamper direct purification of the recombinant protein via *Strep*-tag.

Reagent	**Concentration**
β-mercaptoethanol	20 mM
$CaCl_2$	5 mM
CHAPS	1 %
Ethanol	20 %
Glycerol	50 %
Guanidine HCl	6 M
$MgCl_2$	4 M
NaCl	2 M
Triton X-100	2 %
Tween 20	2 %
Urea	8 M
Imidazole (reduces binding of contaminating proteins)	Up to 20 mM

Table 2. Reagents compatible with 6xHistidine-tag/Ni-NTA interaction successfully used in concentrations up to those given.

The *Strep*/6xHistidine double-tag system was further developed to guarantee purification of full-length recombinant proteins at high purity under standardized conditions which is especially useful for high-throughput attempts where extensive protein characterization is not possible. Recombinant proteins that carry 6xHistidine-tag at the N-terminus and *Strep*-tag II at the C-terminus (or vice versa) are efficiently expressed in *E. coli*, yeast, insect, or mammalian cells. After cell lysis and clearing of the lysate, such recombinant proteins may be initially purified using IMAC (Immobilized metal ion affinity chromatography) based on the 6xHistidine-tag/-Ni-NTA interaction. After elution from the Ni-NTA matrix with imidazole, the recombinant protein (which also carries the *Strep*-tag II epitope) is loaded directly onto a *Strep*-Tactin matrix. No buffer exchange is required. After a short washing step, the recombinant protein is eluted from the *Strep*-Tactin matrix using desthiobiotin. Biotin may also be used which enables protein preparations of higher concentrations but renders the column inactive thus preventing its re-use.

2 Cloning with pEXPR-IBA vectors

Cloning of an arbitrary gene into pEXPR-IBA expression vectors

The pEXPR-IBA vectors multiple cloning sites include many standard unique restriction sites like *Eco*RI or *Bam*HI for the introduction of foreign genes after PCR. However, the reading

frame of the corresponding vector has to be considered if such restriction sites are planned to be used. Using standard unique restriction sites, additional polylinker derived amino acids are appended at the respective end of the recombinant protein. To avoid the fusion of such polylinker derived amino acids pEXPR-IBA vectors offer a general cloning strategy via Type IIS restriction enzymes, *Bsa*I or *Eco*31I (NEB, MBI Fermentas). They allow the precise fusion of the structural gene with the vector encoded functional elements (*Strep*-tag II and, depending on the vector, BM40-signal sequence, protease cleavage site, 6xHistidine-tag, start codon, or stop codon). To accomplish this it is necessary to adapt the structural gene at both ends of the coding region via PCR (s. cloning scheme in the IBA catalogue or at www.iba-go.com). The essential primer sequences to introduce the *Bsa*I restriction site into the PCR fragment for the cloning with a defined pEXPR-IBA vector can be easily determined with our "Primer D'Signer Software" which is free of charge and can be downloaded at our web site. In order to avoid the incorporation of base substitutions, PCR should be performed with a proof reading DNA polymerase (e.g. *Pfu*, MBI Fermentas) using phosphorothioate (PTO) protected primers. The pEXPR-IBA vectors are compatible with the pASK-IBA vectors having the same number (e.g. pASK-IBA3 with pEXPR-IBA3; pASK-IBA5 with pEXPR-IBA5; etc.) which means that one PCR-fragment can be cloned simultaneously into pASK-IBA and pEXPR-IBA via the *Bsa*I cloning strategy. Also transferring genes from pASK-IBA to pEXPR-IBA and vice versa is simple due to compatible restriction sites.

PCR with *Pfu* DNA polymerase

Standard PCR assay; hot-start; PTO protected primers
Mix the following reagents in a 500 µl reaction tube:

		final concentration:	
dNTP (10 mM each)	1 µl	200 µM	
Forward primer (10 µM)	2,5 µl	500 nM	
Reverse primer (10 µM)	2,5 µl	500 nM	
10x buffer (supplier)	5 µl		
Template DNA	X µl	20 to 200 pg/µl	(plasmid DNA)
		0,1 to 1 ng/µl	(cDNA library)
H_2O	ad 50 µl		

Overlay the sample with 50 µl mineral oil or use a cycler with heated lid and heat the sample at 94 °C for 3 min. Add 1 µl *Pfu* DNA polymerase (2,5 u/µl) and start temperature cycling.

Anneal and denature for 30 sec or 1 min. Since the rate of synthesis of *Pfu* is significantly slower than that of *Taq*, the duration of the DNA synthesis step should be doubled when using *pfu* in comparison to protocols referring to the use of *Taq* polymerase (further information can be obtained from the manufacturer Stratagene). The annealing temperature depends on the primer melting temperatures which can be derived by adding the single base melting temperatures of consecutive bases using 4 °C for each GC pairing and 2 °C for each AT pairing (and 1 °C for each GT pairing). Primers should have a theoretical melting temperature between 60 °C and 70 °C (this will be achieved automatically if the "Primer D'Signer Software" is used). PCR annealing should be performed at 55 °C.

If plasmid DNA with an already cloned gene is used as a template, 15 to 20 cycles are usually sufficient, while 30 to 40 cycles are recommended for cDNA libraries as a template. Generally, the number of cycles should be kept as low as possible in order to minimize the possibility of the incorporation of base substitutions. A final 60°C incubation should be performed for 5 min in order to obtain full length products. Samples are stored at 4°C until agarose gel electrophoresis.

Essential parameters for optimization are the annealing temperature, the duration of synthesis and the template concentration.

Cloning of the PCR product via Type IIS restriction enzymes, *Bsa*I or *Eco*31I

First, the PCR product should be purified. The purification step is recommended to create optimal buffer conditions for effective cleavage of the PCR product. If PCR produced a single product, cleaning can be performed using a spin kit (e.g. Biometra order-no. 4100-460B) without prior separation on an agarose gel. Otherwise, a preparative agarose gel is essential for purification. If a spin kit is used and the DNA fragment is eluted in H_2O, *Bsa*I restriction can be performed immediately without any precipitation step.

The pEXPR-IBA vectors can be digested with the isoschizomers *Bsa*I or *Eco*31I. However, both enzymes show different cutting efficiencies in dependence of the DNA source (vector DNA or PCR fragment) and the incubation time. Therefore we performed a comparison of *Bsa*I vs. *Eco*31I and determined the cloning efficiency by determing the resulting colonies after transformation of the ligation reaction into *E. coli* DH5alpha cells. As a result, we recommend to use *Bsa*I for 1 hour or *Eco*31I for 16 hours for the cleavage of both the PCR fragment and the vector.

			pASK-IBA3			
			*Bsa*I		*Eco*31I	
			1 h	16 h	1 h	16 h
PCR fragment	*Bsa*I	1 h	**1208**	**1028**	**265**	**291**
		16 h	**92**	**51**	**22**	**10**
	*Eco*31I	1 h	**77**	**2**	**12**	**8**
		16 h	**1271**	**1228**	**952**	**1140**
no PCR fragment (control)			**0**	**0**	**0**	**0**

Table 3. Determination the cloning efficiency of a PCR fragment into pASK-IBA3 using *Bsa*I or *Eco*RI.
The vector pASK-IBA3 has been digested by *Bsa*I and *Eco*31I for 1 or 16 hours, respectively (see columns). To reduce background the linerized vector was dephosphorylated using shrimp alkaline phosphatase. The DNA has been purified via an agarose gel and was ligated to PCR fragments which have been digested in the same way (see rows). After overnight incubation at 16°C the ligation reaction was transformed into DH5alpha cells and plated onto LB/ampicillin plates. The resulting colonies were determined and are indicated in bold.

Protocol

For restriction digest of the **PCR fragment** add 5 µl 10x *Bsa*I (or *Eco*31I) restriction buffer to the spin eluate, respectively.
Add H_2O and restriction enzyme to 50 µl, using 10 to 20 units of the enzyme per µg DNA. Overlay with mineral oil or use a Thermo Cycler with heated lid and incubate at 50 °C with *Bsa*I for 1 h (or at 37°C with *Eco*31I for 16 h).

For restriction digest of the **vector** incubate 1-2 µg vector DNA with 10 to 20 units *Bsa*I at 50 °C for 1 hour (or *Eco*31I at 37 °C for 16 hours).
To reduce background after ligation which may result from re-ligated vector, dephosphorylate linerized vector DNA with phosphatase (e.g. shrimp alkaline phosphatase from USB) according to the manufacturers recommendations.

After restriction, the desired vector fragment is purified using a preparative agarose gel with subsequent spin purification whereas the PCR fragment may be purified using the spin kit without prior agarose gel separation. 10 % of the eluates are applied on an analytical agarose gel together with a DNA standard for quantification. Finally the fragments are ligated in a typical assay:

Protocol:
100 ng digested vector fragment
Digested PCR fragment in 3 times molar excess
Buffer for ligation
1 unit T4 DNA ligase
H_2O ad 20 µl
Incubate overnight at 16 °C and store the sample at 4 °C until transformation.

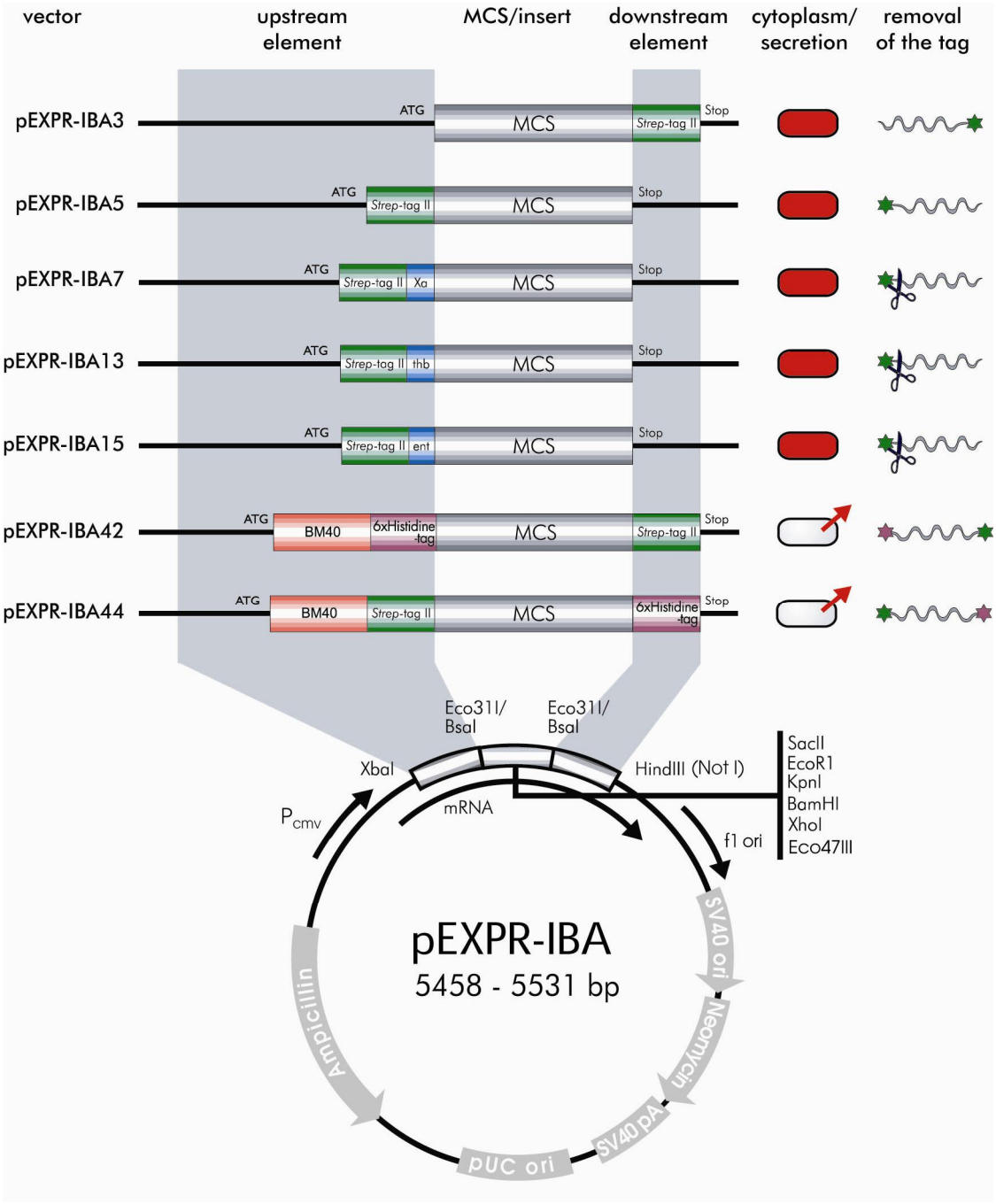
Simultaneously, perform the same ligation assay without the addition of PCR fragment for quantifying background reactions. After transformation and screening for a putative correct clone by DNA mini preparation (Biometra order no. 4100-450B) and subsequent restriction analysis, proceed to DNA sequencing. The sequencing primers are also suitable for cycle sequencing.

Sequencing primers for pEXPR-IBA vectors (order-no. 5-0000-123):

Forward:	5′-GAGAACCCACTGCTTACTGGC-3′
Reverse:	5′-TAGAAGGCACAGTCGAGG-3′

2.1 Expression cassettes of pEXPR-IBA vectors

2.1.1 Overview of pEXPR-IBA vectors



Sequences flanking the *XbaI*/*Hin*dIII restriction sites

```
 CAAT                                TATA                                forward primer
GCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTA


                   XbaI       HindIII NotI                                        reverse primer
ATACGACTCACTATAGGGTCTAGA‖EC*‖AAGCTTGCGGCCGCAGATCTAGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTAGTT

*EC = expression cassette
```

2.1.2 Multiple Cloning Sites

pEXPR-IBA3

```
                Eco31I PshAI                                                   PshAI Eco31I
XbaI            BsaI SacII    EcoRI       KpnI     BamHI XhoI                        BsaI Eco47III                           HindIII
TCTAGACCCACaatgGGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTTGGAGCCACCCGCAGTTCGAAAAATAATAAGCTT
            MetGlyAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaTrpSerHisProGlnPheGluLysEnd
                                                                                            Link       Strep-tag II
```

pEXPR-IBA5

```
                                                   Eco31I PshAI                                                   PshAI Eco31I
XbaI           NheI                                BsaI SacII     EcoRI       KpnI     BamHI XhoI                       BsaI   EcoRV      HindIII
TCTAGACCCACCATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTAACTAAGCTT
            MetAlaSerTrpSerHisProGlnPheGluLysGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                Link       Strep-tag II       Link  ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
                                                     AspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd
```

pEXPR-IBA7

```
                                                          Eco31I PshAI                                                   PshAI Eco31I
XbaI           NheI                                       BsaI SacII     EcoRI       KpnI     BamHI XhoI                       BsaI   EcoRV      HindIII
TCTAGACCCACCATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAAATCGAAGGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTAACTAAGCTT
            MetAlaSerTrpSerHisProGlnPheGluLysIleGluGlyArgArgAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd
                Link       Strep-tag II       Factor Xa   GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                                                           ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
```

pEXPR-IBA13

```
                                                                                   Eco31I PshAI                                                  PshAI Eco31I
XbaI           NheI                                                                BsaI SacII    EcoRI       KpnI     BamHI XhoI                       BsaI   EcoRV      HindIII
TCTAGACCCACCATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAATCTGGTGGTGGTGGTGGTCTGGTTCCGCGTGGctccCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTAACTAAGCTT
            MetAlaSerTrpSerHisProGlnPheGluLysSerGlyGlyGlyGlyGlyLeuValProArgGlySerArgAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd
                Link       Strep-tag II           Spacer            Thrombin       GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                                                                                    ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
```

pEXPR-IBA15

```
                                                                          Eco31I PshAI                                                  PshAI Eco31I
XbaI           NheI                                                       BsaI SacII    EcoRI       KpnI     BamHI XhoI                       BsaI   EcoRV      HindIII
TCTAGACCCACCATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGGCGCCGACGACGACGACAAGGGctccCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTAACTAAGCTT
            MetAlaSerTrpSerHisProGlnPheGluLysGlyAlaAspAspAspAspLysGlySerArgAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd
                Link       Strep-tag II           Enterokinase      Link  GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                                                                           ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
```

pEXPR-IBA42

```
                                                                Eco31I PshAI                                             PshAI Eco31I
XbaI                        NheI                                BsaI SacII   EcoRI      KpnI    BamHI XhoI                    BsaI  Eco47III                        HindIII
TCTAGACCCACCATGAGG./.GCTCTGGCAGCTAGCAGAGGATCGCATCACCATCACCATCACGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTTGGAGCCACCCGCAGTTCGAAAAATAATAAGCTT
           MetArg./.AlaLeuAlaAlaSerArgGlySerHisHisHisHisHisHisGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspPrLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaTrpSerHisProGlnPheGluLysEnd
                  BM40       Link        6xHis-tag         Link                                                                      Link      Strep-tag II
```

pEXPR-IBA44

```
                                                             Eco31I PshAI                                             PshAI Eco31I
XbaI                        NheI                             BsaI SacII   EcoRI      KpnI    BamHI XhoI                     BsaI                                     HindIII
TCTAGACCCACCATGAGG./.GCTCTGGCAGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAggccTGAGAGGATCGCATCACCATCACCATCACTAATAAGCTT
           MetArg./.AlaLeuAlaAlaSerTrpSerHisProGlnPheGluLysGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerGlyLeuArgGlySerHisHisHisHisHisHisEnd
                  BM40       Link      Strep-tag II     Link                                                                       Link         6xHis-tag
```

Please note: Restriction sites in bold cut twice and are useful for precise and oriented insertion of the recombinant gene by one cleavage reaction only. All the regions denoted with "link" carry a restriction site which may be useful for cutting out the recombinant gene for e.g. subcloning into pASK-IBA vectors for bacterial expression. The restriction enzyme cleavage site is not mentioned at the "link" region when such enzyme has at least one further site in the vector.

3 Expression

pEXPR-IBA vectors are designed for high-level stable as well as non-replicative transient expression in most mammalian cells.

3.1 Transient expression in mammalian cells

When the nucleotide sequence is confirmed, prepare enough DNA for the mammalian cell transfection to be carried out. The efficiency of transfection will vary depending on a number of parameters like:

- DNA purity
- host cell line used
- transfection method

Choose a method that will give the highest efficiency for the chosen cell line. Protocols for transfection can be found in [6]. An alternative method is presented below (3.1.1 page 12).

Depending on cell type and nature of the protein, expression levels of 0.1 -1 μg protein can be achieved per million cells (when low expression levels or low transfection efficiency are expected, cell numbers should be increased accordingly), usually sufficient for detection with a specific antibody in total lysates (see chapter 5, page 16). Once expression is verified, transfection of approximately 10^9 cells in general allows expression and purification of protein from mammalian cells in the milligram range. Some proteins however, can not be expressed at high levels[1]. Cell lines latently infected with SV40 or expressing SV40 large T antigen like COS-1 and COS-7 cells allow episomal replication and thus longer expression periods.

Material and important notes

To estimate the number of plates of cells to be transfected, a few common examples are given below.

Cell line	Cell density (cells per cm^2)	Required for 10^9 cells
COS-7	3×10^4	64 x 500 cm^2 plates
CHO-K1	$1{,}25 \times 10^5$	16 x 500 cm^2 plates
HEK-293	$2{,}3 \times 10^5$	9 x 500 cm^2 plates

3.1.1 Transfection

A large number of methods have been establishes for transient transfection. They differ in efficiency, cost and time requirements depending on the cell type used [6]. Among these, magnet assisted transfection (MATra) is a new, easy-to-handle and highly efficient method to transfect cells in culture. Using this new technique nucleic acids, such as plasmid DNA,

[1] In some cases proteins can not be expressed at high levels, especially when they are toxic for the cells. Please refer to section 3.3 on page 13 for hints.

oligonucleotides or siRNA, are in a first step associated with magnetic particles. Exploiting magnetic force the full nucleic acid dose is then rapidly drawn towards and delivered into the target cells leading to efficient transfection. Further Information on MATra is available at www.magnet-assisted-transfection.com

3.2 Stable expression in mammalian cells

pEXPR-IBA vectors contain a neomycin resistance gene to allow for selection of clones that have integrated the pEXPR-IBA DNA stably into their genome. Neomycin concentrations required need to be determined for each cell line (in advance). Determining expression levels in addition to Neomycin resistance is highly recommended. Depending on cell type and nature of the protein expression levels of 0.1-1 μg protein can be achieved per million cells, usually sufficient for detection with a specific antibody in total lysates (see chapter 5, page 16). Some proteins however, can not be expressed at high levels. Please refer to "troubleshooting - expression" in the next section.

3.3 Trouble shooting – Expression

Possible causes for low expression levels and suggested solutions

Problem	**Possible Cause**	**Comments and Suggestions**
Low expression levels or no expression	Sequence error, mutation	Verify sequence and reading frame
	Protein is toxic	Some proteins are inhibiting cell growth or induce apoptosis. In some cases signaling-inactive forms can be expressed at high levels.
	Protein lacks co-factors	Try different cell types, when available cell types related to those endogenously expressing the protein in vivo. Known co-factors can be added to lysis buffer (e.g. metal ions).
Protein is secreted	Signal sequence present	Remove all signal sequences from the coding region.

4 Preparation of soluble cell extracts

4.1 Preparation of soluble extracts after cytoplasmic expression of *Strep*-tag fusion proteins

Material and important notes

- In general, addition of protease inhibitors is recommended. These are available against specific enzymes like trypsin or pepsin inhibitors or in cocktails.
- If activity of enzymes needs to be preserved specific inhibitors may be required like phosphatase inhibitors for kinases and other signaling molecules.
- Reducing agents may be added to prevent disulfide cross-linking.
- Lysis buffers need to be tested for any expressed protein. *Strep*-tag:*Strep*-Tactin binding is compatible with many reagents and detergents (see Table 1 on page 5).

Buffers:

- Buffer W: (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- It is recommended to work without EDTA when metalloproteins have been expressed.
- 5x SDS-PAGE sample buffer: 0.25 M Tris·Cl, pH 8.0; 25% glycerol; 7.5% SDS, 0.25 mg/ml bromophenolblue; 12.5% v/v mercaptoethanol

1. **Chill buffer W at 4°C.**

2. **Thaw the cell pellet for 15 minutes on ice and resuspend the cells in buffer W at 4 ml per 10^9 cells.**

3. **Lyse cells by repeated freeze/thaw cycles.**
   Freeze cells in liquid nitrogen and thaw in a 37°C water bath. Repeat cycle five times.

4. **Shear DNA by passing the lysate through a 18-gauge needle four times. (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 instead.**

5. **Centrifuge lysate at 3,000 x *g* for 15 minutes at 4°C to pellet the cellular debris.**
   A certain proportion of the cellular protein, including the *Strep*-tag protein, may remain insoluble and will be located in the pellet. More complete recovery of the tagged protein needs optimization. E.g. mild detergents (Table 1) or higher ionic strength (e.g. 500 mM NaCl) may help.

6. **Add 5 µl 5x SDS-PAGE sample buffer to 20 µl supernatant and store at -20°C for SDS-PAGE analysis.**

7. **Proceed to protocols for *Strep*-tag protein purification under native conditions (see protocol 6 page 21).**

4.2 Preparation of conditioned supernatant after secreted expression of *Strep*/6xHistidine-tag fusion proteins

In addition to the *Strep*-tag, we have introduced the 6xHistidine-tag in some vectors creating double tag proteins. In a first purification step the 6xHistidine-tag serves for concentrating the recombinant protein out of the medium. As a secondary effect any biotin, which may be present in the medium, is removed. Since biotin binds to *Strep*-Tactin in competition to the *Strep*-tag, biotin in the medium would hamper direct purification of the recombinant protein via *Strep*-tag.

Material and important notes

- In general, supernatants of transfected cells can be directly loaded on Ni-NTA cartridges after addition of 1M Tris pH 8, 3M NaCl, 100mM imidazol to a final concentration of 100 mM Tris pH 8, 300 mM NaCl, 10mM imidazol to shift the pH and adjusting ionic strength and after clearing the supernatant by centrifugation (see 7.2, page 33)
- Very dilute supernatants may need to be concentrated by ultrafiltration or ammonium sulfate precipitation before loading, especially when they are intended to be purified using gravity flow columns. This step can also be used to change the buffer to a pH of 8 required for the purification (see protocols 7 on page 30)

For adherent cells:

1. **Pipet supernatant off the cells and centrifuge at 3,000 x *g* for 15 minutes at 4°C to remove the cellular debris. Proceed to step 2.**

For suspension cells:

1. **Centrifuge cell suspension at 750 x *g* for 10 minutes at 4°C to remove cells and cellular debris.**

2. **For SDS-PAGE analysis: Take 2 ml of the supernatant and concentrate 100-fold. Add 5 µl 5x SDS-PAGE sample buffer to 20 µl supernatant and store at -20°C until analysis.**

3. **Adjust pH (and ionic strength) of supernatant to purification conditions, i.e. by addition of 1/10th volume of 1M Tris/HCl, 3M NaCl, 100mM imidazol pH 8. Dilute samples should be concentrated prior to column purification by ultrafiltration. During this step the buffer change could be performed by adding the 10-fold volume of Ni-NTA Lysis Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8) to the concentrate.**

4. **Proceed to protocols for Strep/6xHistidine-tagged protein purification under native conditions (see protocols 7 on page 30).**
   If the target protein concentration is low, it is recommended to use a Ni-NTA cartridge of appropriate capacity connected to a FPLC workstation because large volumes are difficult to handle via syringe operated cartridges or gravity flow columns described under the above mentioned protocols. The second purification via the *Strep*-tag can then be performed via gravity flow or syringe operated cartridges by transferring the eluate of the Ni-NTA cartridge onto a *Strep*-Tactin cartridge or gravity flow column as described in the respective protocols.

5 Detection of *Strep*-tag fusion proteins

5.1 Detection of *Strep*-tag fusion proteins with *Strep*-Tactin Alkaline Phosphatase (AP) conjugate

Material and important notes

- PBS buffer: 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4
- PBS blocking buffer: PBS buffer with 3 % BSA and 0.5 % v/v Tween 20
- PBS-Tween buffer: PBS buffer with 0.1 % v/v Tween 20
- Reaction buffer: 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris·Cl, pH 8.8
- NTB solution: 7.5 % w/v nitrotetrazolium blue in 70 % v/v dimethylformamid
- BCIP solution: 5 % w/v 5-bromo-4-chloro-3-indolyl-phosphate in dimethyl-formamid
- *Strep*-tag protein ladder can be used as positive control (cat. no. 2-1011-100).
- For blocking biotinylated proteins use Biotin Blocking Buffer (cat. no. 2-0501-002).

1. **Perform SDS-PAGE and electrotransfer of the protein to an appropriate membrane.**

   We recommend to use a nitrocellulose membrane.

2. **Block the membrane with 20 ml PBS-blocking buffer. Incubate 1 h (room temperature; with gentle shaking) or overnight in the refrigerator (4°C).**

   Do not use milk powder for blocking, because milk is one of the richest sources of biotin!

3. **Wash 3 times with 20 ml PBS-Tween buffer (each step: 5 minutes, room temperature, gentle shaking).**

4. **After the last washing step, add 10 ml PBS-Tween buffer to the membrane.**

5. **Optional: Before detection of *Strep*-tag proteins (step 6) add 20 µl Biotin Blocking Buffer (10 minutes, room temperature, gentle shaking).**

   Endogenous biotinylated proteins are specifically blocked.

6. **Add 2.5 µl *Strep*-Tactin alkaline phosphatase conjugate (1:4000). Incubate 60 minutes at room temperature, gentle shaking.**

7. **Wash 2 times with PBS-Tween buffer (each step: 1 minute, room temperature, gentle shaking).**

8. **Wash 2 times with PBS-buffer (each step: 1 minute, room temperature, gentle shaking).**

9. **Transfer membrane in 20 ml reaction buffer and add 10 µl NBT solution and 60 µl BCIP solution.**

10. **Proceed with the chromogenic reaction under shaking until optimal signal:background ratio is achieved.**

11. **Stop reaction by washing several times with distilled H_2O.**

12. **Air dry the membrane and store it in the dark.**

5.2 Detection of *Strep*-tag proteins with *Strep*-Tactin horse radish peroxidase (HRP) conjugates

Material and important notes

- PBS buffer: 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4
- PBS-blocking buffer: PBS buffer with 3 % BSA and 0.5 % v/v Tween 20
- PBS-Tween buffer: PBS buffer with 0.1 % v/v Tween 20
- Chloronaphtol solution: 3 % w/v 4-chloro-1-naphtol in methanol
- H_2O_2 solution: 30 % v/v H_2O_2
- *Strep*-tag protein ladder can be used as positive control (cat. no. 2-1011-100).
- For blocking biotinylated proteins use Biotin Blocking Buffer (cat. no. 2-0501-002).

1. **After SDS-PAGE and electrotransfer of the protein to an appropriate membrane block the membrane with 20 ml PBS-blocking buffer. Incubate: 1 h (room temperature; with gentle shaking) or overnight (4°C).**

   Do not use milk powder for blocking, because milk is one of the richest sources of biotin!
   We recommend to use a nitrocellulose membrane.

2. **Wash 3 times with 20 ml PBS-Tween buffer (each step: 5 minutes, room temperature, gentle shaking).**

3. **After the last washing step, add 10 ml PBS-Tween buffer to the membrane.**

4. **Optional: Before detection of *Strep*-tag proteins (step 5), add 20 µl Biotin Blocking Buffer (10 minutes, room temperature, gentle shaking).**

   Endogenous biotinylated proteins are specifically blocked.

5. **Add 2.5 µl *Strep*-Tactin horse horse radish peroxidase conjugate (1:4000). Incubate 60 minutes at room temperature, gentle shaking.**

6. **Wash 2 times with PBS-Tween buffer (each step: 1 minute, room temperature, gentle shaking).**

7. **Wash 2 times with PBS-buffer (each step: 1 minute, room temperature, gentle shaking).**

8. **Transfer membrane in 20 ml PBS buffer, add 200 µl chloronaphtol solution and 20 µl H_20_2 solution.**

9. **Perform the chromogenic reaction under shaking.**

10. **Stop reaction by washing several times with distilled H_2O.**

11. **Air dry the membrane and store it in the dark.**

5.3 Detection of *Strep*-tag proteins with the *Strep*-tag II specific monoclonal antibody

Material and important notes

- Use *Strep*-tag II purified monoclonal antibody solution (e.g.100 µg/ml in PBS buffer) or crude cell culture supernatant solution
- PBS buffer: 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4
- PBS blocking buffer: PBS buffer with 3 % BSA and 0.5 % v/v Tween 20
- PBS-Tween buffer: PBS buffer with 0.1 % v/v Tween 20
- Chloronaphtol solution: 3 % w/v 4-chloro-1-naphtol in methanol
- H_2O_2 solution: 30 % v/v H_2O_2
- Rabbit anti-mouse antibodies (e.g. DAKO cat. no. P0161)
- *Strep*-tag protein ladder can be used as positive control (cat. no. 2-1011-100).

1. **After SDS-PAGE and electrotransfer of the protein to an appropriate membrane.**
   We recommend to use a nitrocellulose membrane.

2. **Block the membrane with 20 ml PBS-blocking buffer. Incubate 1 h (room temperature; with gentle shaking) or overnight (4°C).**

3. **Wash 3 times with 20 ml PBS-Tween buffer (each step: 5 minutes, room temperature, gentle shaking).**

4. **After the last washing step, add 10 ml PBS-Tween buffer to the membrane.**

5. **Add the specific monoclonal antibody to the solution to a final concentration of 0.2 µg/ml.**

(e.g. 2 µl of a specific monoclonal antibody solution (1 mg/ml) for 10 ml reaction volume)

6. **Incubate 60 minutes at room temperature, gentle shaking.**

7. **Wash 3 times with PBS-Tween buffer (each step: 5 minute, room temperature, gentle shaking).**

8. **After last washing step, add 10 ml PBS-Tween buffer to the membrane.**

9. **Add 10 µl rabbit anti mouse antibodies (e.g. DAKO cat. No. P0161) and incubate under shaking for 45 minutes**

10. **Wash 2 times with PBS-Tween buffer (each step: 1 minute, room temperature, gentle shaking).**

11. **Wash 2 times with PBS buffer (each step: 1 minute, room temperature, gentle shaking).**

12. **Transfer membrane in 20 ml PBS buffer, add 200 µl chloronaphtol solution and 20 µl H_20_2 solution.**

13. **Perform the chromogenic reaction under shaking.**

14. **Stop reaction by washing several times with distilled H_2O.**

15. **Air dry the membrane and store it in the dark.**

5.4 Detection of *Strep*-tag proteins with the *Strep*-tag II specific monoclonal antibody conjugated to horse radish peroxidase (HRP)

Material and important notes

- Use *Strep*-tag II purified monoclonal antibody solution at 1:4000
- PBS buffer: 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4
- PBS blocking buffer: PBS buffer with 3 % BSA and 0.5 % v/v Tween 20
- PBS-Tween buffer: PBS buffer with 0.1 % v/v Tween 20
- Chloronaphtol solution: 3 % w/v 4-chloro-1-naphtol in methanol
- H_2O_2 solution: 30 % v/v H_2O_2
- *Strep*-tag protein ladder can be used as positive control (cat. no. 2-1011-100).

1. **After SDS-PAGE and electrotransfer of the protein to an appropriate membrane.**
   We recommend to use a nitrocellulose membrane.

2. **Block the membrane with 20 ml PBS-blocking buffer. Incubate 1 h (room temperature; with gentle shaking) or overnight (4°C).**

3. **Wash 3 times with 20 ml PBS-Tween buffer (each step: 5 minutes, room temperature, gentle shaking).**

4. **After the last washing step, add 10 ml PBS-Tween buffer to the membrane.**

5. **Add the specific monoclonal HRP conjugate at 1:4000**

6. **Incubate 60 minutes at room temperature, gentle shaking.**

7. **Wash 2 times with PBS-Tween buffer (each step: 5 minute, room temperature, gentle shaking).**

8. **Wash 2 times with PBS buffer (each step: 1 minute, room temperature, gentle shaking).**

9. **Transfer membrane in 20 ml PBS buffer, add 200 µl chloronaphtol solution and 20 µl H_20_2 solution.**

10. **Perform the chromogenic reaction under shaking.**

11. **Stop reaction by washing several times with distilled H_2O.**

12. **Air dry the membrane and store it in the dark.**

6 Purification of *Strep*-tag fusion proteins

6.1 Purification of *Strep*-tag fusion proteins using gravity flow columns

Material and important notes

- **CV = column bed volume**
- *Strep*-Tactin Sepharose, Superflow and MacroPrep can be used for gravity flow purification
- Binding capacity of each matrix is 50 - 100 nmol recombinant protein per ml bed volume (100 nmol correspond to 2 mg of a 20 kDa protein)
- Buffer W (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer E (elution buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8
- 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. For more information see Table 1 on page 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 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.

1. Equilibrate the Strep-Tactin column with 2 CVs Buffer W.

Storage buffer is removed prior to equilibration.
The column cannot run dry under gravity flow.
Use buffer without EDTA for metalloproteins.

2. Centrifuge soluble extracts (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 soluble extracts to the column.

The volume of the supernatants should be in the range of between 0.5 and 10 CVs (see Table 4 on page 21). Extracts of large volumes with the recombinant protein at low concentration may lead to reduced yields and should be concentrated prior to chromatography.
Concentrated cell extracts are preferred; if quantification is possible, apply cell extract containing between 50 and 100 nmol 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. 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.

6.2 Purification of *Strep*-tag fusion proteins using *Strep*-Tactin Spin Columns

Material and important notes

- The spin column matrix binds up to 4 nmol recombinant *Strep*-tag fusion protein (corresponding to 150 µg of a 37 kDa protein (GAPDH-*Strep*-tag))
- Buffer W (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer BE (elution buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 2 mM D-biotin, pH 8
- The composition of the lysis, wash and elution buffers can be modified to suit the particular application, e.g. by adding a mild detergent, a reducing reagent, protease inhibitors, glycerol or by modifying the ionic strength. The pH should not be lower than 7.5, though. For more information see Table 1 on page 5
- Generally, it is recommended to perform protein purification at 4°C

1. **Centrifuge soluble extracts (13,000 rpm, 5 minutes, 4°C, microfuge).**

   Insoluble aggregates which may have formed after storage may clog the column and thus have to be removed.

2. **Equilibrate the Strep-Tactin Spin Column with 2x 500 µl Buffer W. Centrifuge at each step for 30 seconds at 700 x g (approx. 2000 rpm). Discard the flow-through.**

   This rehydrates the dried *Strep*-Tactin resin for the subsequent use. The spin column should be loaded with the soluble extracts containing *Strep*-tag proteins within 20 min, otherwise the capacity of the column might decrease.
   Use buffer without EDTA for metalloproteins.

3. **Load up to 500 µl supernatant of soluble extracts onto the pre-equilibrated *Strep*-Tactin Spin Column. Centrifuge for 30 seconds at 700 x g (approx. 2000 rpm).**

   Collect the flow-through. Apply 2 µl to an analytical SDS-PAGE.
   Lysates with the recombinant protein at low concentration may lead to reduced yields and should be concentrated prior to chromatography. If quantification is possible, apply a volume of lysate containing between 3 and 5 nmol recombinant *Strep*-tag II fusion protein.
   For very concentrated cell lysates, it may be necessary to extend the centrifugation time to 3-4 minutes.

4. **Wash the column 4 times with 100 µl Buffer W. Centrifuge at each step for 30 seconds at 13,000 rpm.**
   Collect the flow-through. Apply 2 µl of the first washing fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE.

5. **Place the spin column into a fresh 1.5 ml reaction tube and choose one of the following procedures for elution:**

   a. **For maximum protein yield:**
      Elute the recombinant protein by adding 3 times 150µl Buffer BE (Biotin-Elution-Buffer). At each step: First, centrifuge for 30 seconds at 700 x g (approx. 2000 rpm) and finish with 15 seconds at maximum speed. Pool the eluates.

   b. **For maximum protein concentration:**
      Elute the protein with 50µl Buffer BE (Biotin-Elution-Buffer). First, centrifuge for 30 seconds at 700 x g (approx. 2000 rpm) and finish with 15 seconds at maximum speed. Transfer the eluate from the collection tube onto the spin column and repeat the centrifugation step as above to maximize the yield.

6.3 Quick purification of *Strep*-tag fusion proteins using cartridges

Material and important notes

- Cartridges filled with 1 ml or 5 ml *Strep*-Tactin Superflow or MacroPrep are available and can be run under some pressure using syringes or FPLC or HPLC workstations.
- Binding capacity of each matrix is 50 - 100 nmol recombinant protein per ml bed volume (100 nmol of a 20 kDa protein correspond to 2 mg)
- Buffer W (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer E (elution buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8
- 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 Table 1 on page 5.
- 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 (maximally 3 cartridges) to enlarge capacity

Protocol for running a 1 ml cartridge with a syringe.

1. **Fill the cartridge inlet with Buffer W.**

2. **Connect a 10 ml syringe filled with Buffer W. Avoid the inclusion of air bubbles.**

3. **Inject 2 ml Buffer W with a flow rate of 30 drops/min to equilibrate the cartridge**

4. **Centrifuge the soluble extracts (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 soluble extracts.**

6. **Remove the 10 ml syringe used for equilibration.**

7. **Fill the cartridge inlet with Buffer W and connect the cartridge with the soluble extracts.**

8. **Apply the soluble extracts with a flow rate of 20 drops/min.**

9. **Remove the syringe, fill the cartridge inlet with Buffer W, fill a 10 ml syringe with Buffer W and connect the syringe with the cartridge.**

10. **Wash the cartridge with 100 drops Buffer W (corresponding to approx. 5 ml) at a flow rate between 20 and 30 drops/min. 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 cartridge 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 fusion protein with 60 drops Buffer E (corresponding to approx. 3 ml) at a flow rate of 20 drops/min. 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.**

6.4 FPLC purification of *Strep*-tag fusion proteins using *Strep*-Tactin Superflow cartridges or *Strep*-Tactin MacroPrep cartridges

If FPLC equipment is used for protein purification cartridges filled with *Strep*-Tactin Superflow or MacroPrep may be used.

Material and important notes

- **CV = cartridge bed volume**
- Binding capacity of each matrix is 50 - 100 nmol recombinant protein per ml bed volume (100 nmol of a 20 kDa protein correspond to 2 mg)
- Cartridges may be connected in series (maximally 3 cartridges) to enlarge capacity
- Recommended flow rates: 1 ml/min for a 1 ml cartridge; 3 ml/min for a 5 ml cartridge.
- Buffer W (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer E (elution buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8
- 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. For more information see Table 1 on page 5.
- 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.
- The cartridges have a female Luer lock inlet and a male Luer lock outlet.

- Different FPLC adapter sets depending on the system used may be necessary and are available (cat. no. 2-1012-000, 2-1013-000, 2-1014-000, 2-1015-000, see IBA catalog 2003/2004 page 21)

1. Connect top adapter to the cartridge.

2. Equilibrate cartridge with 5 CVs of Buffer W.

The flow rate should not exceed 1 ml/min for 1 ml cartridges and 3 ml/min for 5 ml cartridges.
Monitor elution at 280 nm; the baseline should be stable after washing with 5 CVs.

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, the pressure may exceed the maximal value (25 psi). If necessary reduce viscosity of the extract (see Table 4 on page 21) 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.

6.5 HPLC purification of *Strep*-tag fusion proteins using *Strep*-Tactin POROS columns

Material and important notes

- **CV = column bed volume**
- Binding capacity of *Strep*-Tactin POROS 20 or 50 is 25-50 nmol/ml (1.7 ml ready to use column 40-80 nmol; 50 nmol of a 20 kDa protein correspond to 1 mg)
- Linear flow rate 300-500 cm/h, bead size POROS20=20 µm, POROS50=50 µm
- Column format: 4.6 mmD/100 mmL
- Buffer W (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer E (elution buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8
- 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 Table 1 on page 5.

- The bead structure of POROS 20 or 50 is not recommended for purifying membrane proteins.
- The binding capacity of *Strep*-Tactin POROS is about half of that of other *Strep*-Tactin resins.

1. Equilibrate a 1.7 ml Strep-Tactin POROS column with 3 CVs Buffer W.

2. Inject sample

1 ml cell extract in Buffer W containing between 40 and 80 nmol recombinant *Strep*-tag II fusion protein

3. Wash with 6 CVs Buffer W.

Collect fractions for SDS-PAGE analysis.

4. Elute with 3 CVs Buffer E.

Collect fractions for SDS-PAGE analysis.

6.6 Purification of *Strep*-tag fusion proteins using magnetic beads

Material and important notes

- Beads Basic Buffer: 50 mM Tris·Cl, 150 mM NaCl, 5 mM EDTA, pH 7.8
- Beads Activation Buffer: 0.05 % Tween-20 in Beads Basic Buffer
- Beads Elution Buffer: 10 mM biotin in Beads Basic Buffer
- Beads are not re-useable
- 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. For more information see Table 1 on page 5.

Beads must be activated before use:

1. Place reaction tube with bead suspension (100 µl = 5 mg beads) in a magnetic separator (cat. no. 2-1602-000).
2. Remove supernatant.
3. Wash beads 3 times with 0.5 ml Beads Activation Buffer.
4. Place reaction tube with beads in a magnetic separator.
5. Remove supernatant.
6. Beads are ready-to-use.

1. Centrifuge soluble extracts (13,000 rpm, 4°C, 15 minutes, microfuge), discard precipitate.

2. Filtrate obtained lysate supernatant (0.45 µm filter).

Store filtrated supernatant at 4°C.

3. **Add 40 µl extract and 40 µl Beads Basic Buffer to 5 mg activated beads.**
4. **Incubate 30 - 60 minutes at room temperature, shake periodically.**
5. **Place the reaction tube in a magnetic separator (cat. no. 2-1602-000).**
6. **Remove supernatant.**
7. **Add 100 µl Beads Basic Buffer to the beads.**
8. **Vortex and incubate for 1 - 2 minutes.**

   Do not incubate in the magnetic separator.
9. **Separate beads in the magnetic separator.**
10. **Remove supernatant.**

    Your protein of interest is bound to the magnetic beads.
11. **Repeat step 7 to 10 four times.**
12. **After finishing the washing procedure, add 50 µl Beads Elution Buffer to the magnetic beads.**
13. **Incubate 5 - 10 minutes, vortex periodically.**
14. **Place the tube in a magnetic separator.**
15. **Save supernatant containing the recombinant *Strep*-tag fusion protein.**

6.7 Trouble shooting – *Strep*-tag purification

6.7.1 "No or weak binding to *Strep*-Tactin column"

pH is not correct.	The pH should be > 7.0
Strep-tag II is not present.	Add protease inhibitors during cell lysis.
Strep-tag II is not accessible.	Fuse *Strep*-tag with the other protein terminus; use other linker.
Strep-tag II has been degraded.	Check that the *Strep*-tag is not associated with a portion of the protein that is processed.
Strep-tag II is partially accessible.	Reduce washing volume to 3 CVs.
Strep-Tactin column is inactive.	Check activity with HABA. Add avidin (Biotin Blocking Buffer) if biotin containing extracts are intended to be purified.

6.7.2 "Contaminating proteins"

Contaminants are short forms of the tagged protein.	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 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.	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 X100, 0,1 % Tween, 0.1 % CHAPS, etc).

6.7.3 "Bubbles in the column"

When the column is taken from the cold storage room to the bench, the different temperatures can cause small 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 bubbles from developing 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.

7 Purification of *Strep*/6xHistidine-double-tag fusion proteins

The *Strep*/6xHistidine system (double-tag) was developed to guarantee purification of full-length recombinant proteins at high purity under standardized conditions which is especially useful for high-throughput attempts where extensive protein characterization is not possible. Recombinant proteins that carry the 6xHistidine-tag at the N-terminus and the *Strep*-tag II at the C-terminus (or vice versa) are efficiently expressed in *E. coli*, yeast, insect, or mammalian cells. After cell lysis and clearing of the lysate, such recombinant proteins may be initially purified using IMAC (Immobilized metal ion affinity chromatography) based on the 6xHistidine-tag-Ni-NTA interaction. After elution from the Ni-NTA matrix with imidazole, the recombinant protein (which also carry the *Strep*-tag II epitope) is loaded directly onto a *Strep*-Tactin matrix. No buffer exchange is required. After a short washing step, the recombinant protein is eluted from the *Strep*-Tactin matrix using desthiobiotin. Biotin may also be used which enables protein preparations of higher concentrations but renders the column inactive thus preventing its re-use.

7.1 Gravity flow purification of *Strep*/6xHistidine-tag fusion proteins under native conditions

7.1.1 First step: Ni-NTA chromatography

Material and important notes

- **CV = column bed volume**
- Ni-NTA Superflow and Ni-NTA Sepharose are available for column chromatography.
- The binding capacity of Ni-NTA resin is protein dependent and normally lies between 5 and 10 mg/ml (300-500 nmol of a 20 kDa protein).
- Ni-NTA Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8
- Ni-NTA Wash Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol, pH 8
- Ni-NTA Elution Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol, pH 8
- The composition of the lysis, wash and elution buffers can be modified to suit the particular application, e.g. by adding 0.1% Tween, 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information see Table 1 on page 5.
- 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 columns are stored at 4°C and transferred to room temperature air bubbles may form due to restricted solubility of air at elevated temperatures. Therefore, it is recommended to equilibrate the columns immediately after exposure to higher temperatures with buffer that is equilibrated at such temperatures.

1. **Equilibrate the Ni-NTA column with 2 CVs Ni-NTA Lysis Buffer.**

   Storage buffer is removed.
   The column cannot run dry under gravity flow.

2. **Centrifuge cell supernatant from protocol 4.2 on page 15 (14,000 rpm, 5 minutes, 4°C, microfuge).**

   Insoluble aggregates are removed which otherwise could clog the column.

3. **Transfer supernatant to the column.**

4. **Wash the column 5-8 times with 1 CV Ni-NTA Wash Buffer, after the cell supernatant has completely entered the column.**

   Collect the eluate in 1 CV fractions and 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 Ni-NTA Elution Buffer and collect the eluate in 0.5 CV fractions.**

   20 µl samples of each fraction can be used for SDS-PAGE analysis.
   The purified 6xHistidine-tag fusion protein usually elutes in the 2nd to 5th fraction.

6. **After SDS-PAGE analysis pool the fractions containing the Strep/6xHistidine-tag fusion protein.**

7.1.2 Second step: *Strep*-Tactin chromatography

Material and important notes

- **CV = column bed volume**
- Strep-Tactin Sepharose, Superflow and MacroPrep can be used for gravity flow purification.
- Binding capacity of each matrix is 50 - 100 nmol recombinant protein per ml bed volume (100 nmol correspond to 2 mg of a 20 kDa protein)
- Buffer W (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer E (elution buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8
- 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. For more information see Table 1 on page 5.
- 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 columns are stored at 4°C and transferred to room temperature air bubbles may form due to restricted solubility of air at elevated temperatures. 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)	Volume of the eluate after Ni-NTA column	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 5: Recommended buffer volumes for chromatography on *Strep*-Tactin columns

7. Equilibrate the Strep-Tactin column with 2 CVs Buffer W.

Storage buffer is removed prior to equilibration.
The column cannot run dry under gravity flow.
Use buffer without EDTA for metalloproteins.

8. Add the pooled fractions of Ni-NTA chromatography (from step 6 of the previous protocol) on the column.

The volume of the pooled fractions should be between 0.5 and 10 CVs.

9. Wash the column 5 times with 1 CV Buffer W, after the pooled fractions have completely entered the column.

Collect the eluate in fractions having a size of 1 CV. Apply 20 µl of each fraction to an analytical SDS-PAGE.

10. 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.
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.

7.2 Quick purification of *Strep*/6xHistidine-tag fusion proteins under native conditions using cartridges

7.2.1 First step: Ni-NTA chromatography

Material and important notes

- 1 ml and 5 ml Ni-NTA Superflow cartridges are available.
- The binding capacity of Ni-NTA Superflow is protein dependent and normally lies between 5 and 10 mg/ml (300-500 nmol of a 20 kDa protein).
- Ni-NTA Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8
- Ni-NTA Wash Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol, pH 8
- Ni-NTA Elution Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol, pH 8
- The composition of the lysis, wash and elution buffers can be modified to suit the particular apllication, e.g. by adding 0.1% Tween, 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information see Table 1 on page 5.
- 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 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.

7.2.2 Second step: *Strep*-Tactin chromatography

Material and important notes

- Cartridges filled with 1 ml or 5 ml *Strep*-Tactin Superflow or MacroPrep are available and can be run under pressure using syringes or FPLC or HPLC workstations.
- Binding capacity of each matrix is 50 - 100 nmol recombinant protein per ml bed volume (100 nmol of a 20 kDa protein correspond to 2 mg)
- Buffer W (washing buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer E (elution buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8
- 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. For more information see Table 1 on page 5.
- 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 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 may be connected in series (maximally 3 cartridges) to enlarge capacity

Protocol is for the use of 1 ml cartridges.

1. **Fill the Ni-NTA cartridge inlet with Ni-NTA Lysis Buffer.**

2. **Connect a 10 ml syringe filled with Ni-NTA Lysis Buffer. Avoid the inclusion of air bubbles.**

3. **Inject 2 ml Ni-NTA Lysis Buffer with a flow rate of 30 drops/min to equilibrate the cartridge.**

4. **Centrifuge the supernatants from protocol 4.2 (14,000 rpm, 5 minutes, 4°C, microfuge) containing the Strep/6xHistidine-tag fusion protein to remove aggregates that may have formed during storage.**

5. **Fill a syringe with the appropriate amount of the supernatants.**

6. **Remove the 10 ml syringe used for equilibration.**

7. **Fill the cartridge inlet with Ni-NTA Lysis Buffer and connect the syringe containing the supernatants.**

8. **Apply the supernatants with a flow rate of 20 drops/min.**

9. **Remove the syringe, fill the cartridge inlet with Ni-NTA Wash Buffer, fill a 10 ml syringe with Ni-NTA Wash Buffer and connect with the cartridge.**

10. **Wash the cartridge with 160 drops (corresponding to approx. 8 ml) Ni-NTA Wash Buffer at a flow rate between 20 and 30 drops/min. 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 W8).**

11. **Remove the syringe and fill the cartridge inlet with Ni-NTA Elution Buffer.**

12. **Fill a 5 ml syringe with 4 ml Ni-NTA Elution Buffer.**

    An alternative to imidazole elution is possible. The column must be incubated for 15 minutes with 1 CV EDTA buffer (100 mM Tris·Cl, 150 mM NaCl, 20 mM EDTA, pH 8). After this procedure the displaced protein can be washed out directly **with Buffer W** onto the cartridge filled with Strep-Tactin resin. By this procedure the Nickel will be stripped off the NTA matrix which must be regenerated prior to re-use for purification of a 6xHistidine protein (see protocol 8.2.1, page 38).

13. **Fill the inlet of a *Strep*-Tactin resin cartridge with Buffer W.**

14. Screw the Ni-NTA cartridge onto the *Strep*-Tactin cartridge.

15. Inject Ni-NTA Elution Buffer or Buffer W (if the EDTA buffer has been used) with a flow rate of 20 drops/min.

The *Strep*/6xHistidine-tag fusion protein will bind to the *Strep*-Tactin resin.

16. Screw off the Ni-NTA cartridge.

17. Fill the inlet of the *Strep*-Tactin cartridge with Buffer W.

18. Fill a 10 ml syringe with Buffer W and connect it with the *Strep*-Tactin cartridge.

19. Wash with 100 drops Buffer W at a flow rate between 20 and 30 drops/min. Collect the eluate in fractions of 20 drops and apply 20 µl of each fraction to an analytical SDS-PAGE (fraction W6 to W10).

20. Fill the cartridge inlet with Buffer E.

21. Fill a syringe with 4 ml Buffer E and inject Buffer E with a flow rate of 20 drops/min.

22. Elute with 60 drops Buffer E. Collect the eluate in fractions of 10 drops and apply 20 µl of each fraction to an analytical SDS-PAGE (fraction E1 to E6). The double tag protein usually elutes in the 2nd to 5th fraction.

7.3 Trouble shooting 6xHistidine-tag purification

For problems related to Strep-tag purification please refer to chapter 6.7 page 29.

7.3.1 "Protein does not bind to Ni-NTA"

6xHistidine-tag is not present.	Sequence ligation junctions to ensure that the reading frame is correct. Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal).
6xHistidine-tag is inaccessible.	Move tag to the other terminus of the protein.
6xHistidine-tag has been degraded.	Check that the 6xHistidine-tag is not associated with a portion of the protein that is processed.
Binding conditions incorrect.	Check pH and compositions of all buffers and solutions. The pH values should be checked immediately prior use. Ensure that there are no chelating or reducing agents present, and that the concentration of imidazole is not too high.

7.3.2 "Protein elutes with the Ni-NTA Wash Buffer"

Wash stringency is to high.	Lower the concentration of imidazole or increase the pH slightly.
6xHistidine-tag is partially hidden.	Reduce washing stringency. Purify under denaturing conditions.
Buffer conditions incorrect.	Check pH and composition of Ni-NTA Wash Buffer. Ensure that there are no chelating or reducing agents present.

7.3.3 "Protein precipitates during purification"

Temperature is too low.	Perform purification at room temperature.
Protein forms aggregates.	Try adding solubilization reagents such as 0.1% Triton X-100 or Tween-20, up to 20 mM β-ME, up to 2 M NaCl, or stabilizing cofactors such as Mg^{2+}. These may be necessary in all buffers to maintain protein solubility.

7.3.4 "Protein does not elute"

Elution conditions are to mild (protein may be in an aggregate or multimer form).	Elute with a pH or imidazole step-gradient to determine the optimal elution conditions.
Protein has precipitated in the column.	Elute under denaturing conditions. Perform binding and elution in batch format to avoid high local protein concentrations.

7.3.5 "Protein elutes with contaminants"

Binding and washing conditions are not stringent enough.	Include 10-20 mM imidazole in the binding and wash buffers.
Column is too large.	Reduce the amount of Ni-NTA resin.
Contaminants are associated with tagged protein.	Add β-mercaptoethanol to a maximum of 20 mM to reduce disulfide bonds. Increase salt and/or detergent concentrations in the wash buffer to disrupt nonspecific interactions.
Contaminants are truncated forms of the tagged protein.	Check for possible internal translation starts (C-terminal tag) or premature termination sites (N-terminal tag). Prevent protein degradation during purification by working at 4°C or by including protease inhibitors. Fuse a *Strep*-tag to the other terminus of the protein to select for full length proteins by a two step purification by means of both tags.

7.3.6 "Discoloration of resin"

Nickel ions are removed or reduced.	Ensure that there are no chelating compounds (resin color turns white) or reducing agents (resin color turns brown) present in all buffers.

8 APPENDIX

8.1 Storage and regeneration of *Strep*-Tactin resin

Material and 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.
- Buffer R (regeneration buffer): 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, 1 mM HABA (hydroxy-azophenyl-benzoic acid), pH 8.0
- Buffer W: 100 mM Tris·Cl, 150 mM NaCl, 1 mM EDTA, pH 8
- 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.

8.1.1 Regeneration of gravity flow columns filled with *Strep*-Tactin resin

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.**

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.**

8.1.2 Regeneration of cartridges with *Strep*-Tactin resin

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.**

8.2 Storage and regeneration of Ni-NTA resin

Material and important notes

- Ni-NTA matrices need not be refrigerated.
- After use they should be washed for 30 minutes with 0.5 M NaOH.
- Matrices should be stored in 30% ethanol to inhibit microbial growth.
- Matrices can be stored up to one week in any denaturing buffer.
- We recommended a maximum of 5 runs per column.
- If the color of Ni-NTA resin changes from light blue to brownish-gray, the matrix should be regenerated.
- If the recombinant protein is eluted with EDTA, the matrix must be regenerated.

8.2.1 Regeneration of Ni-NTA material

1. **Wash the column with 2 CV of regeneration buffer (6 M GuHCl, 0.2 M acetic acid).**
2. **Wash the column with 5 CV of H_2O.**
3. **Wash the column with 3 CV of 2% SDS.**
4. **Wash the column with 1 CV of 25% EtOH.**
5. **Wash the column with 1 CV of 50% EtOH.**
6. **Wash the column with 1 CV of 75% EtOH.**
7. **Wash the column with 5 CV of 100% EtOH.**
8. **Wash the column with 1 CV of 75% EtOH.**
9. **Wash the column with 1 CV of 50% EtOH.**
10. **Wash the column with 1 CV of 25% EtOH.**
11. **Wash the column with 1 CV of H_2O.**
12. **Wash the column with 5 CV of 100 mM EDTA, pH8.0.**
13. **Wash the column with H_2O.**
14. **Recharge the column with 2 CV of 100 mM $NiSO_4$.**
15. **Wash the column with 2 CV of H_2O.**
16. **Wash the column with 2 CV of regeneration buffer.**
17. **Equilibrate with 2 CV of a suitable buffer.**

9 Related products

Cat. No.	Product
2-0501-002	Biotin Blocking Buffer; 2 ml
2-1000-001	D-Desthiobiotin; 0.107 g
2-1000-002	D-Desthiobiotin; 1g
2-1000-005	D-Desthiobiotin; 5 g
2-1000-025	D-Desthiobiotin (10x Buffer E); 25 ml
2-1001-003	Strep-Tactin® Column Evaluation Set
2-1001-013	Strep-Tactin® Mini-Column Evaluation Set
2-1002-000	Strep-tag® protein purification buffer set
2-1002-100	Strep-tag® regeneration buffer with HABA; 100 ml
2-1011-100	Strep-tag® Protein Ladder
2-1012-000	M6 adapter set for FPLC
2-1013-000	1/4-28 adapter set for FPLC
2-1014-000	10-32 adapter set for HPLC and Äkta
2-1015-000	1/16 inch adapter set for peristaltic pump tubing
2-1101-000	Strep-tag® Starter Kit
2-1102-000	Strep-tag® Starter Kit 3C
2-1103-000	Strep-tag® Starter Kit "Cartridge" MacroPrep® + adapters
2-1104-000	Strep-tag® Starter Kit "Cartridge" MacroPrep®
2-1105-000	Strep-tag® Starter Kit "Cartridge" Superflow® + adapters
2-1106-000	Strep-tag® Starter Kit "Cartridge" Superflow®
2-1107-000	Strep/His Starter Kit
2-1201-010	Strep-Tactin® Sepharose; 20 ml 50 % suspension
2-1201-025	Strep-Tactin® Sepharose; 50 ml 50 % suspension
2-1201-100	Strep-Tactin® Sepharose; 200 ml 50 % suspension
2-1201-500	Strep-Tactin® Sepharose; 1000 ml 50 % suspension
2-1202-001	Gravity flow Strep-Tactin® Sepharose column; 1 x 1 ml
2-1202-005	Gravity flow Strep-Tactin® Sepharose column; 5 x 1 ml
2-1202-051	Gravity flow Strep-Tactin® Sepharose column; 1 x 5 ml
2-1202-101	Gravity flow Strep-Tactin® Sepharose column; 1 x 10 ml
2-1202-505	Gravity flow Strep-Tactin® Sepharose column; 5 x 0.2 ml
2-1203-001	Strep-Tactin® POROS® 20; 2 ml 50 % suspension
2-1203-002	Strep-Tactin® POROS® 20; 4 ml 50 % suspension
2-1203-005	Strep-Tactin® POROS® 20; 10 ml 50 % suspension
2-1203-010	Strep-Tactin® POROS® 20; 20 ml 50 % suspension
2-1203-017	Ready-to-use Strep-Tactin® POROS® 20 column; 1.7 ml
2-1205-001	Strep-Tactin® POROS® 50; 2 ml 50 % suspension
2-1205-002	Strep-Tactin® POROS® 50; 4 ml 50 % suspension
2-1205-005	Strep-Tactin® POROS® 50; 10 ml 50 % suspension

Cat. No.	Product
2-1205-010	Strep-Tactin® POROS® 50; 20 ml 50 % suspension
2-1205-017	Ready-to-use Strep-Tactin® POROS® 50 column; 1.7 ml
2-1206-010	Strep-Tactin® Superflow®; 20 ml 50 % suspension
2-1206-025	Strep-Tactin® Superflow®; 50 ml 50 % suspension
2-1206-100	Strep-Tactin® Superflow®; 200 ml 50 % suspension
2-1206-500	Strep-Tactin® Superflow®; 1000 ml 50 % suspension
2-1207-001	Gravity flow Strep-Tactin® Superflow® column; 1 x 1 ml
2-1207-005	Gravity flow Strep-Tactin® Superflow® column; 5 x 1 ml
2-1207-051	Gravity flow Strep-Tactin® Superflow® column; 1 x 5 ml
2-1207-101	Gravity flow Strep-Tactin® Superflow® column; 1 x 10 ml
2-1207-505	Gravity flow Strep-Tactin® Superflow® column; 5 x 0.2 ml
2-1211-001	Strep-Tactin® Superflow® cartridge; 1 x 1 ml
2-1211-005	Strep-Tactin® Superflow® cartridge; 5 x 1 ml
2-1212-001	Strep-Tactin® Superflow® cartridge; 1 x 5 ml
2-1212-005	Strep-Tactin® Superflow® cartridge; 5 x 5 ml
2-1501-001	Strep-Tactin® coated microplate; 1 plate
2-1501-005	Strep-Tactin® coated microplate; 5 plates
2-1502-000	Strep-tag® HRP Detection Kit
2-1502-001	Strep-Tactin® HRP conjugate; 0.5 ml
2-1503-000	Strep-tag® AP Detection Kit
2-1503-001	Strep-Tactin® AP conjugate; 0.5 ml
2-1505-010	Strep-Tactin® MacroPrep®; 20 ml 50 % suspension
2-1505-025	Strep-Tactin® MacroPrep®; 50 ml 50 % suspension
2-1505-100	Strep-Tactin® MacroPrep®; 200 ml 50 % suspension
2-1505-500	Strep-Tactin® MacroPrep®; 1000 ml 50 % suspension
2-1506-001	Gravity flow Strep-Tactin® MacroPrep® col.; 1 x 1 ml
2-1506-005	Gravity flow Strep-Tactin® MacroPrep® col.; 5 x 1 ml
2-1506-051	Gravity flow Strep-Tactin® MacroPrep® col.; 1 x 5 ml
2-1506-101	Gravity flow Strep-Tactin® MacroPrep® col.; 1 x 10 ml
2-1506-505	Gravity flow Strep-Tactin® MacroPrep® col.; 5 x 0.2 ml
2-1507-001	Strep-tag II specific monoclonal antibody, purified; 100 µg
2-1508-025	Strep-tag II monocl. antibody, cell supernat. for 25 blots
2-1508-050	Strep-tag II monocl. antibody, cell supernat. for 50 blots
2-1509-001	Strep-tag II specific monoclonal antibody, HRP conjugate
2-1511-001	Strep-Tactin® MacroPrep® cartridge; 1 x 1 ml
2-1511-005	Strep-Tactin® MacroPrep® cartridge; 5 x 1 ml
2-1512-001	Strep-Tactin® MacroPrep® cartridge; 1 x 5 ml
2-1512-005	Strep-Tactin® MacroPrep® cartridge; 5 x 5 ml

Cat. No.	Product
2-1601-000	MagStrep Kit
2-1601-002	MagStrep Beads; 2 ml
2-1601-005	MagStrep Beads; 5 ml
2-1602-000	Magnetic Separator for 24 rxn tubes
2-1700-000	Strep-well HT 25 Purification Starter Kit
2-1701-000	Strep-well HT 50 Purification Starter Kit
2-1702-000	Strep-well HT buffer set for 10 plates
2-1703-000	Strep-well HT buffer set for 25 plates
2-1704-000	Strep-well HT buffer set for 100 plates
2-1705-010	Strep-well HT filter plates; 10 plates
2-1706-010	Strep-well HT wash plates; 10 plates
2-1707-010	Strep-well HT receiver plates; 10 plates
2-1725-010	Strep-well HT 25 Purification Plates; 10 plates
2-1725-025	Strep-well HT 25 Purification Plates; 25 plates
2-1725-100	Strep-well HT 25 Purification Plates; 100 plates
2-1750-010	Strep-well HT 50 Purification Plates; 10 plates
2-1750-025	Strep-well HT 50 Purification Plates; 25 plates
2-1750-100	Strep-well HT 50 Purification Plates; 100 plates
2-1903-000	pEXPR-IBA3, 5 µg
2-1905-000	pEXPR-IBA5, 5 µg
2-1907-000	pEXPR-IBA7, 5 µg
2-1913-000	pEXPR-IBA13, 5 µg
2-1915-000	pEXPR-IBA15 5µg, 5 µg

Cat. No.	Product
2-1942-000	pEXPR-IBA42, 5 µg
2-1944-000	pEXPR-IBA44, 5 µg
2-3201-010	Ni-NTA Sepharose; 50 % suspension; 20 ml
2-3202-001	Ni-NTA Sepharose gravity flow column; 1 ml
2-3202-051	Ni-NTA Sepharose gravity flow column; 5 ml
2-3206-010	Ni-NTA Superflow® ; 50 % suspension; 20 ml
2-3207-001	Ni-NTA Superflow® gravity flow column; 1 ml
2-3207-051	Ni-NTA Superflow® gravity flow column; 5 ml
2-3211-001	Ni-NTA Superflow® cartridge; 1 x 1 ml
2-3211-005	Ni-NTA Superflow® cartridge; 5 x 1 ml
2-3212-001	Ni-NTA Superflow® cartridge; 1 x 5 ml
2-3212-005	Ni-NTA Superflow® cartridge; 5 x 5 ml
5-0000-121	Forward sequencing primer for pEXPR-IBA; 1 nmol
5-0000-122	Reverse sequencing primer for pEXPR -IBA; 1 nmol
5-0000-123	Forw. and rev. seq. primers for pEXPR -IBA; 1 nmol each
7-2001-020	MATra-A Reagent for 200 µg nucleic acids
7-2001-100	MATra-A Reagent for 1000 µg nucleic acids
7-2002-020	MATra-S Immobilizer up to 7 Mio. cells
7-2002-100	MATra-S Immobilizer up to 35 Mio. cells
7-2003-020	MA Lipofection Enhancer up to 200 µg nucleic acids
7-2003-100	MA Lipofection Enhancer up to 1000 µg nucleic acids
7-2004-000	96 Magnet Plate

10 References

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

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