Expression and purification of proteins using *Strep*-tag and/or 6xHistidine-tag

A comprehensive manual

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IBA Headquarters
IBA GmbH
Rudolf-Wissell-Str. 28
D-37079 Göttingen
Germany
Tel: +49 (0) 551-50672-0
Fax: +49 (0) 551-50672-181
info@iba-go.com
www.iba-go.com

IBA US Distribution Center
1328 Ashby Road
Olivette, MO 63132
Tel. 1-877-IBA-GmbH (1-877-422-4624)
Fax 1-888-531-6813
info@iba-go.com
www.iba-go.com

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Content

1 Introduction

1.1 *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 purifing metallo- and membrane proteins, large proteins and protein complexes. Binding capacity depends on the *Strep*-Tactin matrices (25 - 100 nmol/ml using standard *Strep*-Tactin resins and up to 500 nmol/ml using *Strep*-Tactin Superflow High Capacity) 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 http://www.iba-go.com/.

	Concentration
Reduction Agents	
DTT	50 mM
β-mercaptoethanol	50 mM
Non-Ionic Detergents	
C_8E_4 Octyltetraoxyethylene	max. 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-β-D-maltoside	0.35 %
DDM; N-dodecyl-β-D-maltoside	2 %
Digitonin	0,5 %
LM; N-dodecyl-β-D-maltoside	0.007 %
NG; N-nonyl-β-D-glucopyranoside	0.2 %
NP40; Nonidet P40	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	0.5 %
DDAO; N-decyl-N,N-dimethylamine-N-oxide	0.034 %
Fos-Choline-12	0,1 %
LDAO; N-dodecyl-N,N-dimethylamine-N-oxide	0.13 %

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 vary for other proteins.

1.2 6xHistidine-tag/Ni-NTA system

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]. NTA, which has four chelating sites for nickel ions, binds nickel more tightly than metal-chelating purification systems like IDA which have only three sites available for interaction with metal ions. The extra chelating site prevents nickel-ion leaching, thus providing greater binding capacity and high-purity protein preparations. Under denaturing conditions this system is the system of choice because the 6xHistidine-tag/Ni-NTA interaction tolerates high concentrations of urea and guanidine (see Table 2). Under physiological conditions host proteins with histidine stretches or host proteins containing metal ions may contaminate the protein preparation. Working with 20 mM imidazole can reduce this problem. However, we recommend the *Strep*-tag or the *Strep*/6xHistidine-system for physiological conditions.

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.

1.3 *Strep*/6xHistidine system (double-tag)

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 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, preferably on Strep-Tactin Superflow High Capacity. 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 results in a protein preparation of higher concentration but renders the column inactive thereby preventing its re-use.

2 Cloning with pASK-IBA and pPR-IBA vectors

Cloning of an arbitrary gene into pASK-IBA and pPR-IBA expression vectors

The pASK-IBA and pPR-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. In some vectors with N-terminal *Strep*-tag II, *Strep*-tag II is followed by the linker sequence 5'-GGCGCC. This sequence is recognized by 3 different restriction enzymes generating 5'-overhangs. Cleavage with the suitable enzyme and, if necessary, a subsequent filling reaction makes the production of blunt ends in all reading frames possible. 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 pASK-IBA and pPR-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, *Omp*A-signal sequence, protease cleavage site, Histidine-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). In order to avoid the incorporation of base substitutions, PCR should be performed with a proof reading DNA polymerase (e.g. *Pfu*, Stratagene) using phosphorothioate protected primers. The essential primer sequences to introduce the *Bsa*I restriction site into the PCR fragment for the cloning with a certain pASK-IBA or pPR-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. Alternatively to individual cloning, the use of IBA's generic StarGate cloning system is less complex, faster and offers more options (other tags like FLAG, GST and other promoters for mammalian, baculo or yeast expression). Please refer to www.stargate-cloning.com.

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 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 Design 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 pASK-IBA and pPR-IBA vectors can be digested with the isoschizomers *Bsa*I or *Eco*31I. However, both enzymes show different cutting efficiencies regarding 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 counting the resulting colonies after transformation of the ligation reaction into 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. Determining the cloning efficiency of a PCR fragment into pASK-IBA3 using *Bsa*I or *Eco*RI. (Counted colonies are indicated in bold.) 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 are determined.

Protocol (for optimal combination see Table 3)

For restriction digest of the **PCR fragment** add 5 µl 10x *Eco*31I (or *Bsa*I) 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 and incubate at 37°C with *Eco*31I for 16 h (or at 50 °C with *Bsa*I for 1 h), respectively.

For restriction digest of the **vector** incubate 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 results from re-ligated vector either incubate with *Pst*I for further 30 min at 37 °C or 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.

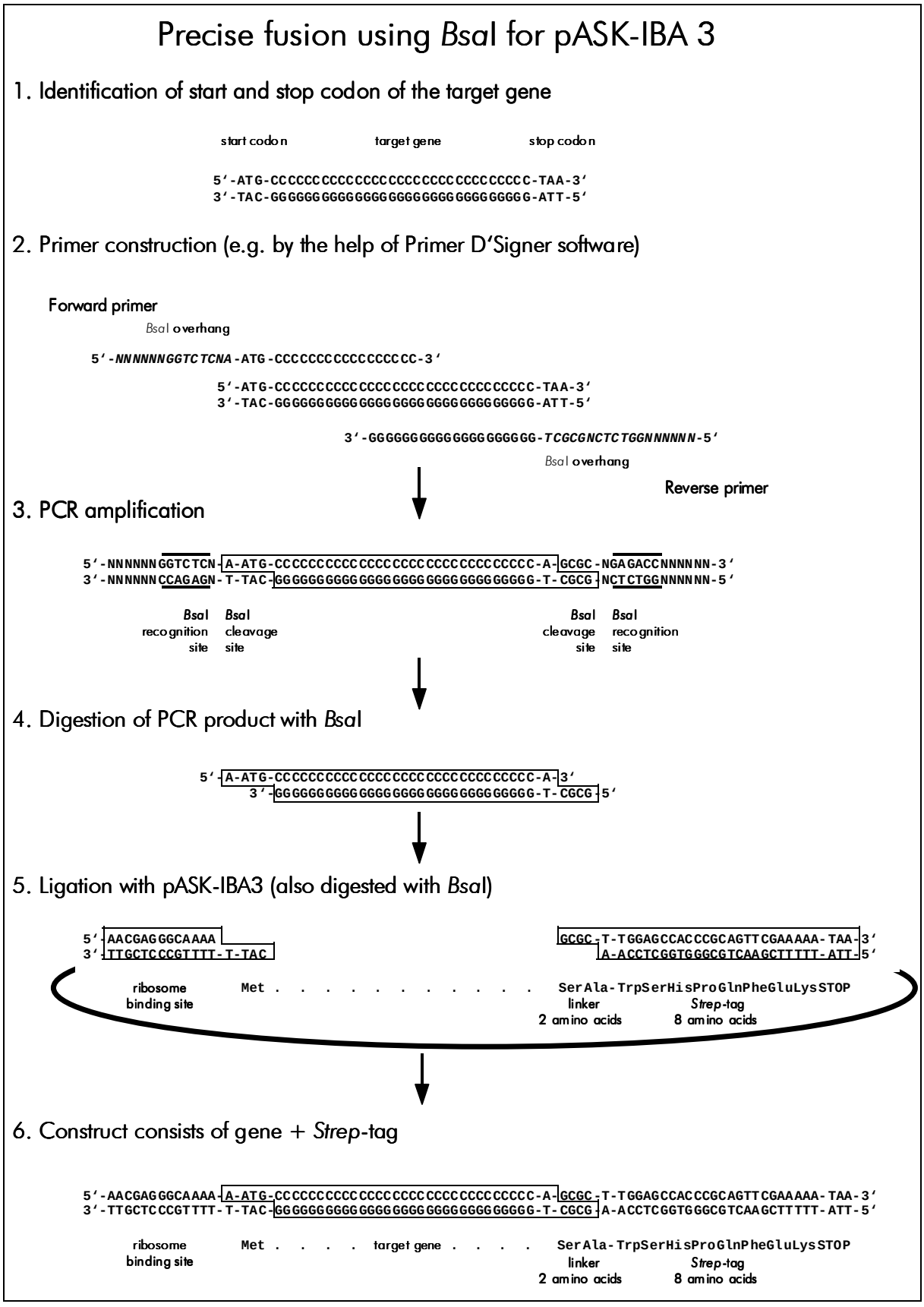
Sequencing primers for pASK-IBA vectors (order-no. 5-0000-103):

Forward:	5′-GAGTTATTTTACCACTCCCT-3′
Reverse:	5′-CGCAGTAGCGGTAAACG-3′

Sequencing primers for pPR-IBA vectors (order-no. 5-0000-113):

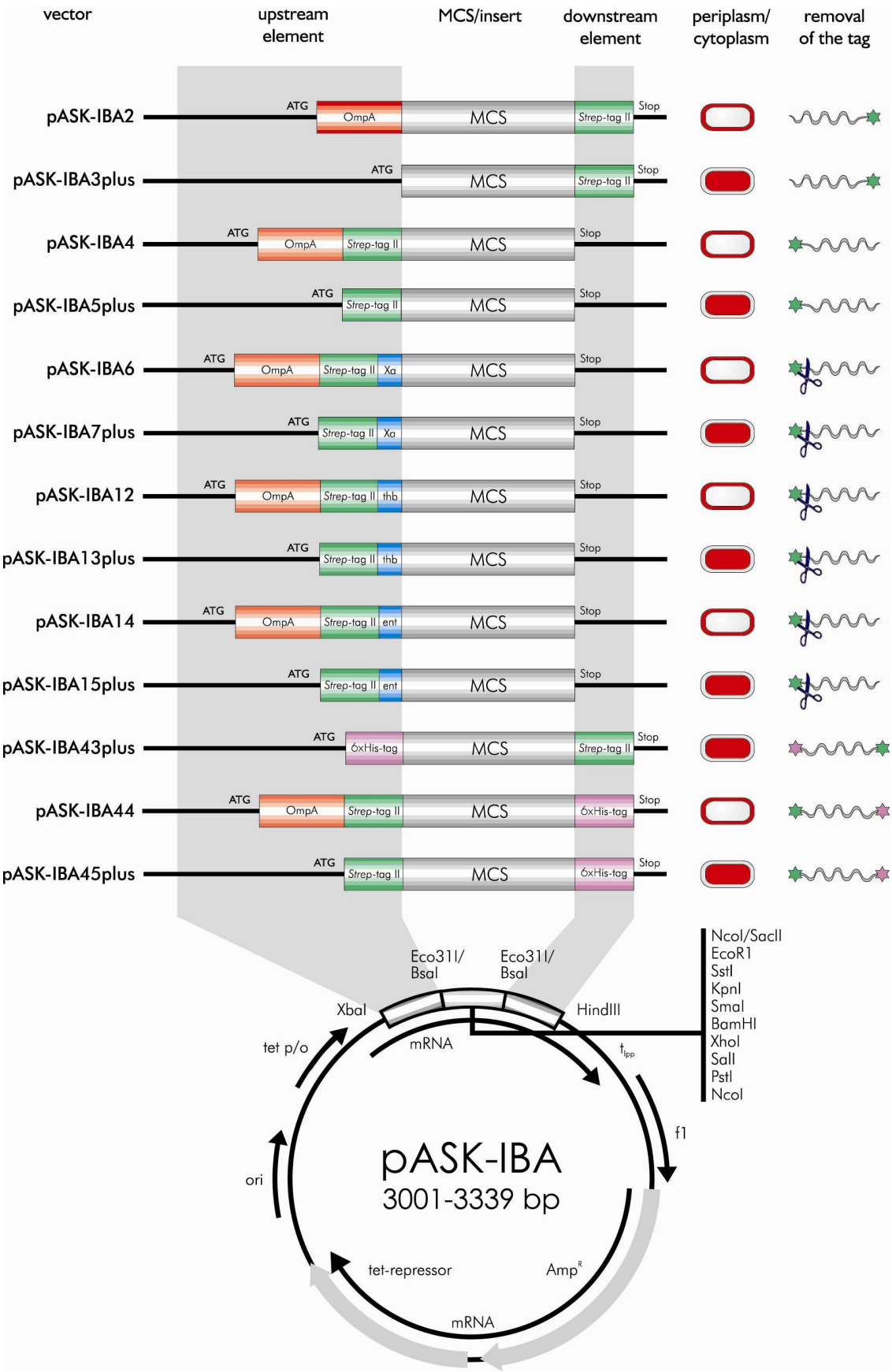
Forward:	5′-TAATACGACTCACTATAGGG-3′
Reverse:	5′-TAGTTATTGCTCAGCGGTGG-3′

2.1 Cloning scheme for the use of *BsaI* or *Eco31I*



2.2 Expression cassettes of pASK-IBA and pPR-IBA vectors

2.2.1 Overview of pASK-IBA vectors with Strep-tag and double tag



Multiple cloning sites of pASK-IBA plasmids

```
pASK-IBA2
                                          Eco31I PshAI                                                 PshAI Eco31I
XbaI      RBS                             BsaI NcoI BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI Eco47III            BstBI        HindIII
TCTAGATAACGAGGGCAAAAAATGAAA../..GCGCAggccGGAGACCATGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTTGGAGCCACCCGCAGTTCGAAAAATAATAAGCTT
                     MetLys../..AlaGlnAlaGlyAspHisGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaTrpSerHisProGlnPheGluLysEnd
                            OmpA                                                                                     Link       Strep-tag II


pASK-IBA3plus
                                              Eco31I PshAI                                                  PshAI Eco31I
XbaI                        RBS               BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI Eco47III           BstBI        HindIII
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAaatgGGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTTGGAGCCACCCGCAGTTCGAAAAATAATAAGCTT
                                          MetGlyAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaTrpSerHisProGlnPheGluLysEnd
                                                                                                                         Link       Strep-tag II


pASK-IBA4
                                                                       BbeI
                                                                       EheI
                                                                       NarI  Eco31I PshAI                                                  PshAI Eco31I
XbaI      RBS                            NheI                 BstBI    KasI  BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI EcoRV     HindIII
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                            OmpA          Link       Strep-tag II       Link  ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
                                                                               AspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd


pASK-IBA5plus
                                                                           BbeI
                                                                           EheI
                                                                           NarI  Eco31I PshAI                                                  PshAI Eco31I
XbaI                        RBS              NheI                 BstBI    KasI  BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI EcoRV     HindIII
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                                              Link       Strep-tag II       Link  ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
                                                                                   AspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd


pASK-IBA6
                                                                              BbeI
                                                                              EheI
                                                                              NarI  Eco31I PshAI                                                  PshAI Eco31I
XbaI      RBS                            NheI                 BstBI           KasI  BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI EcoRV     HindIII
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                            OmpA          Link       Strep-tag II       Factor Xa   GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                                                                                     ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd


pASK-IBA7plus
                                                                                  BbeI
                                                                                  EheI
                                                                                  NarI  Eco31I PshAI                                                  PshAI Eco31I
XbaI                        RBS              NheI                 BstBI           KasI  BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI EcoRV     HindIII
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                                              Link       Strep-tag II       Factor Xa   GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
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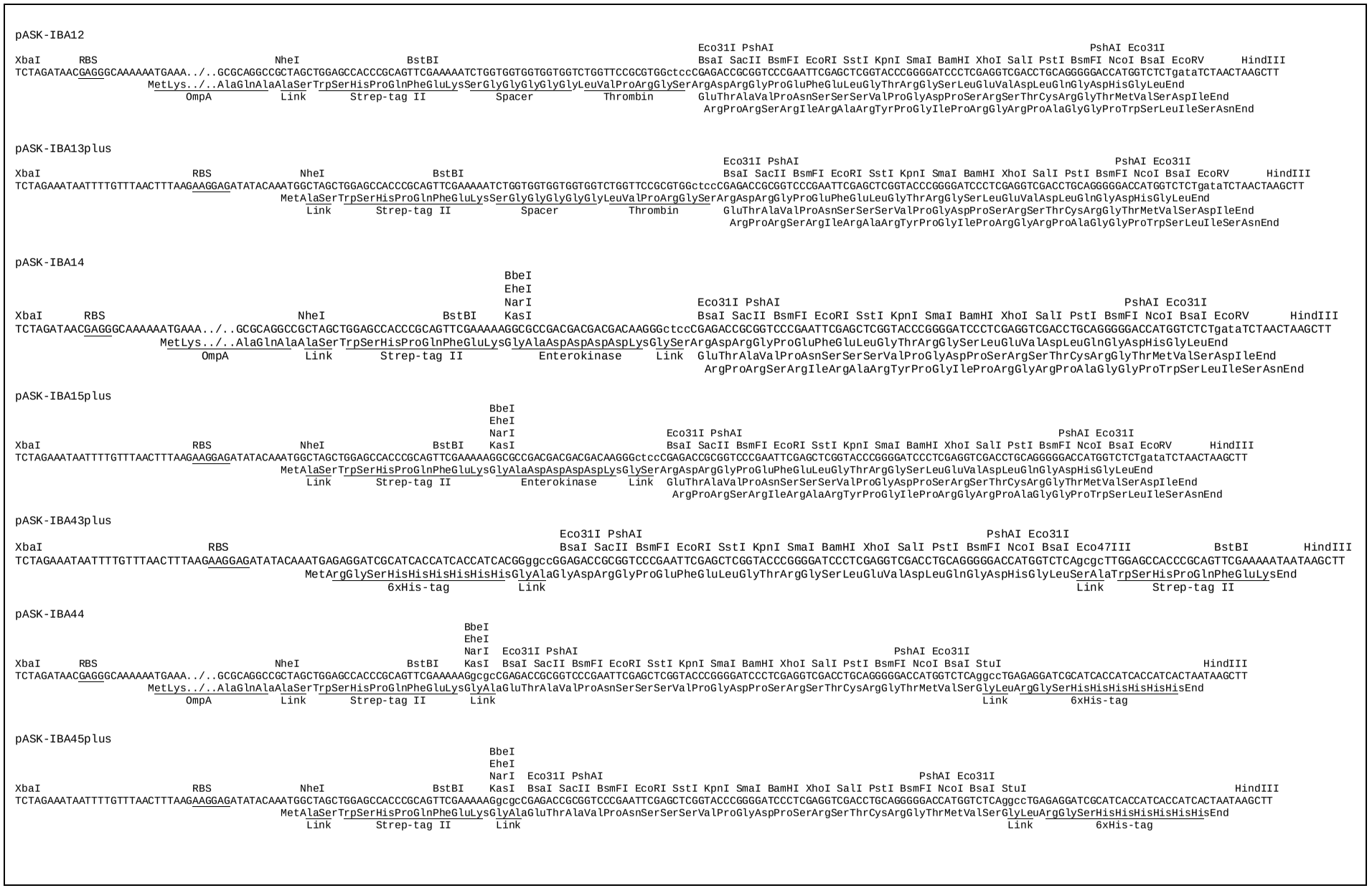
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OmpA Link Strep-tag II Spacer Thrombin GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
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Link Strep-tag II Spacer Thrombin GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
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EheI
NarI
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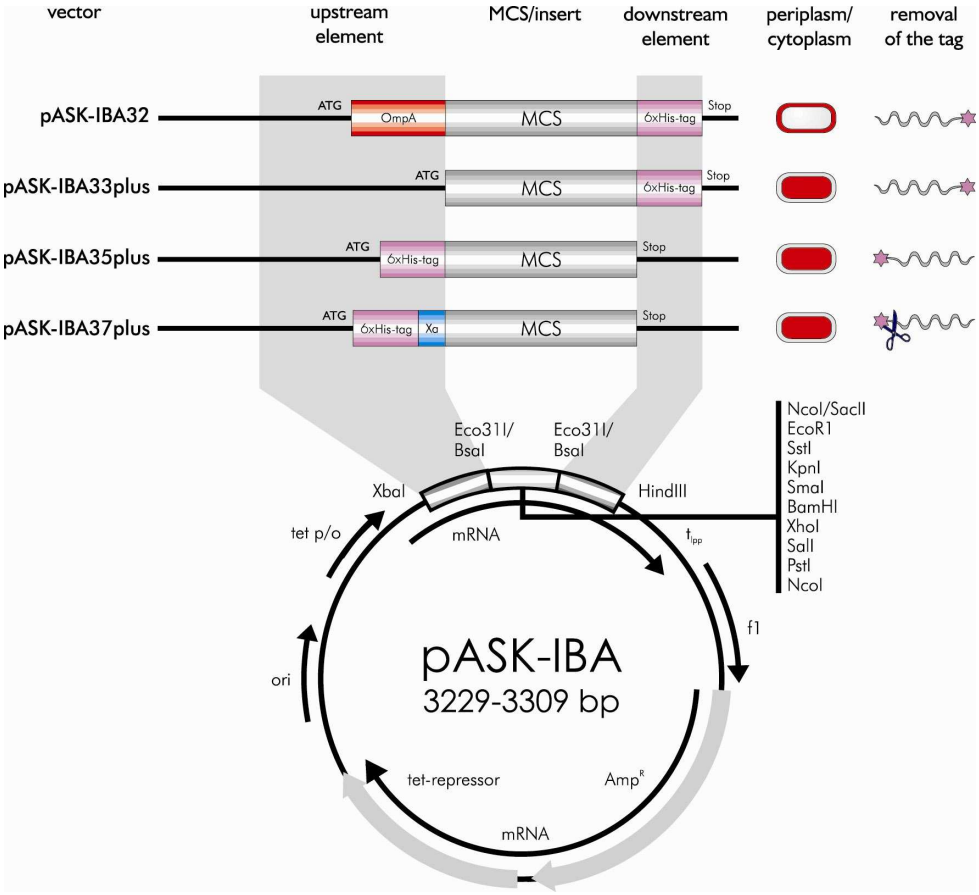
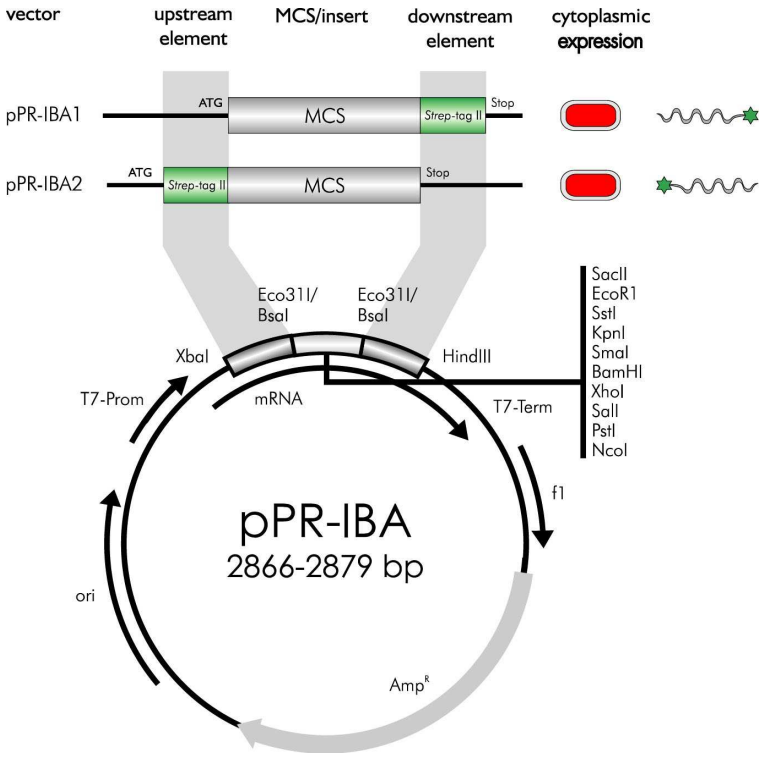
BbeI
EheI
NarI Eco31I PshAI
PshAI Eco31I
XbaI RBS NheI BstBI KasI BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI StuI HindIII
TCTAGATAACGAGGGCAAAAAATGAAA../..GCGCAGGCCGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGgcgccGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAggccTGAGAGGATCGCATCACCATCACCATCACTAATAAGCTT
MetLys../..AlaGlnAlaAlaSerTrpSerHisProGlnPheGluLysGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerGlyLeuArgGlySerHisHisHisHisHisHisEnd
OmpA Link Strep-tag II Link Link 6xHis-tag
pASK-IBA45plus
BbeI
EheI
NarI Eco31I PshAI
PshAI Eco31I
XbaI RBS NheI BstBI KasI BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI StuI HindIII
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAAATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGgcgccGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAggccTGAGAGGATCGCATCACCATCACCATCACTAATAAGCTT
MetAlaSerTrpSerHisProGlnPheGluLysGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerGlyLeuArgGlySerHisHisHisHisHisHisEnd
Link Strep-tag II Link Link 6xHis-tag

2.2.2 Overview of pASK-IBA vectors with 6xHistidine-tag



2.2.3 Overview of pPR-IBA vectors



Multiple cloning sites of pASK-IBA and pPR-IBA plasmids

```
pASK-IBA32
                                          Eco31I PshAI                                                PshAI Eco31I
XbaI      RBS                             BsaI NcoI BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI Eco47III                             HindIII
TCTAGATAACGAGGGCAAAAAATGAAA../..GCGCAggccGGAGACCATGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTAGAGGATCGCATCACCATCACCATCACTAATAAGCTT
                     MetLys../..AlaGlnAlaGlyAspHisGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaArgGlySerHisHisHisHisHisHisEnd
                           OmpA                                                                                         Link          6xHis-tag


pASK-IBA33plus
                                              Eco31I PshAI                                                PshAI Eco31I
XbaI                        RBS               BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI Eco47III                             HindIII
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAaatgGGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTAGAGGATCGCATCACCATCACCATCACTAATAAGCTT
                                          MetGlyAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaArgGlySerHisHisHisHisHisHisEnd
                                                                                                                         Link          6xHis-tag

pASK-IBA35plus
                                                                              BbeI
                                                                              EheI
                                                                              NarI  Eco31I PshAI                                                PshAI Eco31I
XbaI                        RBS              NheI                             KasI  BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI EcoRV      HindIII
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAAATGGCTAGCAGAGGATCGCATCACCATCACCATCACGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTAACTAAGCTT
                                          MetAlaSerArgGlySerHisHisHisHisHisHisGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                                              Link          6xHis-tag          Link  ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
                                                                                      AspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd

pASK-IBA37plus
                                                                                     BbeI
                                                                                     EheI
                                                                                     NarI  Eco31I PshAI                                                PshAI Eco31I
XbaI                        RBS              NheI                                    KasI  BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI EcoRV      HindIII
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAAATGGCTAGCAGAGGATCGCATCACCATCACCATCACATCGAAGGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTAACTAAGCTT
                                          MetAlaSerArgGlySerHisHisHisHisHisHisIleGluGlyArgArgAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd
                                              Link          6xHis-tag          Factor Xa   GluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                                                                                            ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd


pPR-IBA1
                                              Eco31I PshAI                                                PshAI Eco31I
XbaI                         RBS              BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI Eco47III            BstBI        HindIII
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAaatgGGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTTGGAGCCACCCGCAGTTCGAAAAATAATAAGCTT
                                          MetGlyAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaTrpSerHisProGlnPheGluLysEnd
                                                                                                                         Link       Strep-tag II

pPR-IBA2
                                                                           BbeI
                                                                           EheI
                                                                           NarI  Eco31I PshAI                                                PshAI Eco31I
XbaI                         RBS             NheI                 BstBI    KasI  BsaI SacII BsmFI EcoRI SstI KpnI SmaI BamHI XhoI SalI PstI BsmFI NcoI BsaI EcoRV      HindIII
TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGGcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTAACTAAGCTT
                                          MetAlaSerTrpSerHisProGlnPheGluLysGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd
                                              Link       Strep-tag II       Link  ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd
                                                                                   AspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd
```

3 Expression

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

The production of a heterologous protein is often accompanied by an impaired growth of *E. coli* cells. Consequently, regulation of heterologous biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor. If the foreign protein is cytotoxic, even the production of minute quantities can result in a dramatic selection against the *E. coli* cells which harbour the expression plasmid. In such cases, tight repression of the promoter is required. Synthesis of the gene product is then switched on in a controlled manner simply by adding a chemical inducer. The *Strep*-tag II expression vectors that carry the promoter/operator region from the *tet*A resistance gene are the state-of-the-art solution for such an inducible expression system [6,7]. The strength of the *tet*A promoter is comparable with that of the *lac*-UV5 promoter (nearly 25 % activity of the T7 promoter).

It can be fully induced by adding anhydrotetracycline at a concentration that is not antibiotically effective (200 ng/ml). The constitutive expression of the *tet* repressor gene, which is also encoded on the expression plasmids, guarantees the repression of the promoter in the absence of the inducer. In a Western blot, no expression is detectable under these conditions [6,8]. In contrast to the *lac* promoter, which is susceptible to catabolite repression (cAMP-level, metabolic state) and chromosomally encoded repressor molecules, the *tet*A promoter/operator is not coupled to any cellular regulation mechanisms. Therefore, when using the *tet* system, there are basically no restrictions in the choice of culture medium or *E. coli* expression strain. For example, glucose minimal media and even the bacterial strain XL1-Blue, which carries an episomal copy of the tetracycline resistance gene, can be used for expression.

Material and important notes

- Ampicillin stock solution (pASK-IBA vectors except pASK-IBA2C, 3C, 4C, 5C, 6C, 7C): 100 mg/ml in H_2O, sterile filtered. Store in aliquots at -20°C.
- Chloramphenicol stock solution (pASK-IBA2C, 3C, 4C, 5C, 6C, 7C, recommended for fermentation at high cell densities): 30 mg/ml in ethanol. 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, 150 mM NaCl, 1 mM EDTA
- 5x SDS-PAGE sample buffer: 0.250 M Tris·Cl, pH 8.0; 25% glycerol; 7,5% SDS, 0.25 mg/ml bromophenolblue; 12.5% v/v mercaptoethanol
- 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.

1. **Preculture: Inoculate 2 ml of LB medium containing 100 µg/ml ampicillin (pASK-IBA plasmids except 2C to 7C) or 30 µg/ml chloramphenicol (pASK-IBA2C to 7C) with a fresh colony harbouring the pASK-IBA expression plasmid and shake overnight (200 rpm) at 37°C.**
   The colony should not be older than 1 week.

   Do not inoculate from glycerol stocks.

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

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

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

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

5. **When OD_{550} equals 0.5-0.6, add 10 µl of anhydrotetracycline solution.**
   The yield of soluble, functional protein may be substantially increased, particularly in case of periplasmic expression, by lowering the growth temperature to between 22°C and 30°C.

6. **Shake for 3 hours at 200 rpm.**
   Overnight induction may increase protein yields in some cases.

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

8. **Proceed to "Preparation of Cleared Lysates" (page 22) or store cell pellet at -20°C.**

3.2 Expression in *E. coli* with the T7-system (pPR-IBA vectors)

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

Expression of the target genes is induced by providing a source of T7 RNA polymerase in the E. coli host cell (DE3 lysogen). This is accomplished by using e.g. 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 [9,10].

Material and important notes

- Ampicillin stock solution: 100 mg/ml in H_2O, sterile filtered. Store in aliquots at -20°C.
- Buffer W: 100 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM EDTA
- LB medium: 10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl
- 20% glucose, sterile filtered
- IPTG stock solution (1M): 238 mg/ml in H_2O, sterile filtered. Store in aliquots at -20°C.
- 5x SDS-PAGE sample buffer: 0.250 M Tris·Cl, pH 8.0 ; 25% glycerol; 7.5% SDS, 0.25 mg/ml bromophenolblue; 12.5% v/v mercaptoethanol

1. **Preculture: Inoculate 2 ml of LB medium containing 100 µg/ml ampicillin with a fresh colony harbouring the pPR-IBA expression plasmid and shake overnight (200 rpm) at 37°C.**
   The colony should not be older than 1 week.

   Do not inoculate from glycerol stocks.

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

   In case of toxic proteins, the leakiness of the lacUV5 promoter and the resulting expression may lead to cell death or to the selection of non-productive mutants. Add 2 % glucose and/or use pLysS or pLysE cotransformants in such cases [9].

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

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

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

5. **When OD_{550} equals 0.5-0.6, add 50 µl of IPTG stock solution (0.5 mM end concentration).**

6. **Shake for 3 hours at 200 rpm.**
   Overnight induction may increase protein yields in some cases.

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

9. **Proceed to "Preparation of Cleared Lysates" (page 23) or store cell pellet at -20°C.**

3.3 Expression with other systems

Successful bacterial expression has the advantage of obtaining the expression product in a short time at low costs. Nevertheless, there are proteins which cannot be expressed in *E. coli*. Yeast, insect, mammalian or plant cells are alternative expression hosts for such proteins. Both *Strep*-tag and 6xHistidine-tag can be expressed in eukaryotic cells. IBA's pEXPR-IBA line of vectors is designed for mammalian expression via the CMV promoter and they are compatible in many cases with pASK-IBA vectors so that a PCR fragment can be cloned in parallel for both mammalian and bacterial expression. Vectors for expression of *Strep*-tag II fusion proteins in yeast or insect cells are commercialized by IBA within the StarGate system only (www.stargate-cloning.com).

Please notice that eukaryotic cultivation media may contain significant amounts of biotin. Media for insect cell cultivation contain e. g. about 800 nmol biotin per liter medium. That means if proteins are secreted to the medium, biotin must be masked by the addition of avidin or biotin should be removed by dialysis or gel filtration or by performing ammonium sulfate precipitation prior to *Strep*-Tactin chromatography. Ammonium sulfate precipitation, if applicable, has the advantage that the recombinant protein will be concentrated at the same time. Please contact IBA in case of any questions.

3.4 Trouble shooting – Expression

Problem	Comments and suggestions
No or low expression	Check the culture condition (e.g. IPTG, anhydrotetracycline, antibiotics) Check vector (sequence, frame) Check whether the protein is found in the insoluble fraction. Reduction of temperature during cultivation may solve this problem (e.g. 16°C, 22°C, 26°C, 30°C). Use another expression system (e.g. T7 promoter instead of Tet promoter, see page 19). Use eukaryotic cells for expression (yeast, insect or mammalian cells)
Protein is degraded	Use protease deficient *E. coli* strains. If degradation occurs during cell lysis, add protease inhibitor If the protein is small (<10 kDa), consider adding a terminal carrier protein. Lower temperature during induction can reduce the problem. Secretion of the recombinant protein to the periplasmic space can reduce the problem.
Protein is secreted	Remove all signal sequences from the coding region.
Inclusion bodies are formed: Protein is insoluble	Reduce expression level by modifying growth and induction conditions, e.g.: lower culturing temperature (16°C, 22°C, 26°C, 30°C), Use another expression system (e.g. Tet promoter instead of T7 promoter, see page 17).

4 Preparation of Cleared Lysates

4.1 Preparation of cleared lysate after cytoplasmic expression of *Strep*-tag fusion proteins

Material and important notes

- Buffer W: 100 mM Tris/HCl pH8, 150 mM NaCl, 1 mM EDTA
- It is recommended to work without EDTA when metalloproteins have been expressed.
- *Strep*-tag:*Strep*-Tactin binding is compatible with many reagents and detergents (see Table 1 on page 6).
- 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. **Resuspend the pellet of a 100 ml culture in 1 ml Buffer W.**

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

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

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

6. **Centrifuge the suspension at 13000 rpm (microfuge) for 15 minutes at 4°C.**
   Insoluble cell components are sedimented. If the recombinant protein forms inclusion bodies it will be present in the sediment.

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

8. **Proceed to protocols for *Strep*-tag protein purification under native conditions (see protocols 6.1 to 6.4).**
   Strep-tag/*Strep*-Tactin affinity purification should not be performed discontinuously in batch mode which would result in significantly reduced protein purity and yield in comparison to column chromatography. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. If batch purification with the Strep-tag system is attempted, the use of Strep-Tactin Superflow High Capacity in combination with the One-STrEP-tag is recommended.

4.2 Preparation of cleared lysate after periplasmic expression of *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 following translocation by *E. coli* leader peptidase. In order to purify proteins secreted into the periplasmic space using *Strep*-Tactin technology [11,12], the *Strep*-tag can be fused to the C- or N-terminus using pASK-IBA2, 2C, 4, 4C, 6, 6C, 12, 14, 44.

Material and important notes

- Buffer P for the release of the periplasmic content: 100 mM Tris/HCl pH8, 500 mM sucrose, 1 mM EDTA
- It is recommended to work with 2 mg/ml polymyxin B sulfate instead of 1 mM EDTA when metalloproteins are isolated.
- Buffer conditions and binding conditions of *Strep*-tag on *Strep*-Tactin are compatible with many reagents (see Table 1).

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 [13]. 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 5 x SDS-PAGE sample buffer. Store at -20°C. The whole sample must be incubated in an ultrasonic bath for 15 minutes to reduce the chromosomal DNA to small pieces and should be heated to 70°C for 10 minutes prior to SDS-PAGE.

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

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

7. **Proceed to protocols for *Strep*-tag protein purification under native conditions (see protocols 6.1 to 6.4).**
   Strep-tag/*Strep*-Tactin affinity purification should not be performed discontinuously in batch mode which would result in significantly reduced protein purity and yield in comparison to column chromatography. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. If batch purification with the Strep-tag system is attempted, the use of Strep-Tactin Superflow High Capacity in combination with the One-STrEP-tag is recommended.

4.3 Preparation of cleared lysate after cytoplasmic expression of 6xHistidine-tag proteins under native conditions

Material and important notes

- Ni-NTA Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8
- Lysozyme
- 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. **Thaw the cell pellet for 15 minutes on ice and resuspend the cells in Ni-NTA Lysis Buffer at 2-5 ml per gram wet weight.**
   Ni-NTA Lysis Buffer contains 10 mM imidazole to minimize binding of untagged, histidine rich contaminating proteins and increase purity with fewer wash steps.
   If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1-5 mM. With 6xHistidine-tag proteins exhibiting high binding affinities, the imidazole concentration can be increased to 20 mM.

2. **Add lysozyme to 1 mg/ml and incubate on ice for 30 minutes.**

3. **Sonicate on ice.**
   Use six 10 second bursts at 200-300 W with a 10 second cooling period between each burst.
   Use a sonicator equipped with a microtip.

4. **(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.**

5. **Centrifuge lysate at 10,000 x *g* for 20-30 minutes at 4°C to pellet the cellular debris.**
   A certain proportion of the cellular protein, including the 6xHistidine-tag protein, may remain insoluble and will be located in the pellet. For more complete recovery of the tagged protein, this material must be solubilized using denaturing conditions.

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 6xHistidine-tagged protein purification under native conditions (see protocols 7.1 to 7.3).**

4.4 Preparation of cleared lysate after periplasmic expression of 6xHistidine-tag fusion proteins

Periplasmic proteins are proteins secreted into the periplasmic space located between the outer and inner membrane of *E. coli*. Proper secretion is possible only when the protein of interest has an N-terminal signal peptide which is cleaved following translocation. In order to purify proteins secreted into the periplasmic space [11,12] using Ni-NTA technology, the 6xHistidine-tag must be fused to the C-terminus of the target protein. N-terminal 6xHistidine-tags might be processed with the transit signal.

Material and important notes

- Sucrose buffer: 30 mM Tris·Cl, 20% sucrose, pH 8.0
- 500 mM EDTA solution
- 5 mM $MgSO_4$ solution, ice-cold
- Ni-NTA Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8

1. **Resuspend cell pellet in sucrose buffer at 80 ml per gram wet weight.**
2. **Keep the cells on ice and add 500 mM EDTA solution dropwise to 1 mM.**
3. **Incubate the cells on ice for 5-10 minutes with gentle agitation.**
4. **Centrifuge the cell suspension at 8000 x *g* for 20 minutes at 4°C.**
5. **Remove all the supernatant, and resuspend the pellet in the same volume of ice-cold 5mM $MgSO_4$ solution.**
6. **Shake or stir for 10 minutes in an ice bath.**
7. **Centrifuge at 8000 x *g* for 20 minutes at 4°C.**
   The supernatant is the osmotic shock fluid containing periplasmic proteins.
8. **Dialyse supernatant extensively against Ni-NTA Lysis Buffer before continuing with the purification.**
9. **Proceed to protocols for 6xHistidine-tag proteins purification under native conditions (see protocols 7.1 to 7.3).**

4.5 Preparation of cleared lysate of 6xHistidine-tag proteins under denaturing conditions

Material and important notes

- 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
- Buffer B: 100 mM NaH_2PO4, 10 mM Tris·Cl, 8 M urea, pH 8.0
- Cells can be lysed in either 6 M GuHCl or 8 M urea. It is preferable to lyse the cells in the milder denaturant, urea, so that the cell lysate can be analyzed directly by SDS-PAGE. GuHCl is a more efficient solubilization and cell lysis reagent, however, and may be required to solubilize some proteins.

1. **Thaw the cell pellet for 15 minutes on ice and resuspend with Buffer B at 5 ml per gram wet weight.**

2. **Stir cells for 15-60 minutes at room temperature or lyse them by gently vortexing, taking care to avoid foaming.**
   Lysis is complete when the solution becomes translucent.

3. **Centrifuge lysate at 10,000 x g for 20-30 minutes at room temperature to pellet the cellular debris.**
   Save supernatant (cleared lysate).

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

5. **Proceed to protocols for 6xHistidine-tag protein purification under denaturing conditions (see protocols 7.5 to 7.9).**

4.6 Preparation of cleared lysate after cytoplasmic expression of *Strep*/6xHistidine-tag fusion proteins under native conditions

Material and important notes

- Ni-NTA Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8
- Lysozyme
- 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

1. **Thaw the cell pellet for 15 minutes on ice and resuspend the cells with Ni-NTA Lysis Buffer at 2-5 ml per gram wet weight.**
   Ni-NTA Lysis Buffer contains 10 mM imidazole to minimize binding of untagged, contaminating proteins and increase purity with fewer wash steps. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1-5 mM.

2. **Add lysozyme to 1 mg/ml and incubate on ice for 30 minutes.**

3. **Sonicate on ice.**
   Use six 10 second bursts at 200-300 W with a 10 second cooling period between each burst.
   Use a sonicator equipped with a microtip.

4. **(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.**

5. **Centrifuge lysate at 10,000 x *g* for 20-30 minutes at 4°C to pellet the cellular debris.**

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/6xHistidine-tag protein purification under native conditions (see protocols 8.1 to 8.2).**
   Strep-tag/*Strep*-Tactin affinity purification should not be performed discontinuously in batch mode which would result in significantly reduced protein purity and yield in comparison to column chromatography. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. If batch purification with the Strep-tag system is attempted, the use of Strep-Tactin Superflow High Capacity in combination with the One-STrEP-tag is recommended.

4.7 Preparation of cleared lysate after periplasmic expression of Strep/6xHistidine-tag fusion proteins

Periplasmic proteins are proteins secreted into the periplasmic space located between the outer and inner membrane of *E. coli*. Proper secretion is possible only when the protein of interest has an N-terminal signal peptide which is cleaved following translocation. In order to purify proteins secreted into the periplasmic space using Ni-NTA technology, the 6xHistidine-tag must be fused to the C-terminus of the target protein. N-terminal 6xHistidine-tag will be processed with the transit signal. In contrast to 6xHistidine-tag, *Strep*-tag at the N-terminus has no influence on the transit signal [14].

Material and important notes

- Sucrose buffer: 30 mM Tris·Cl, 20% sucrose, pH 8.0
- 500 mM EDTA solution
- 5 mM $MgSO_4$ solution, ice-cold
- Ni-NTA Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8

1. **Resuspend cell pellet in sucrose buffer at 80 ml per gram wet weight.**

2. **Keep the cells on ice and add 500 mM EDTA solution dropwise to 1 mM.**

3. **Incubate the cells on ice for 5-10 minutes with gentle agitation.**

4. **Centrifuge the cell suspension at 8000 x *g* for 20 minutes at 4°C.**

5. **Remove all the supernatant, and resuspend the pellet in the same volume (as used in step 1.) of ice-cold 5 mM $MgSO_4$ solution.**

6. **Shake or stir for 10 minutes in an ice bath.**

7. **Centrifuge at 8000 x *g* for 20 minutes at 4°C.**
   The supernatant is the osmotic shock fluid containing periplasmic proteins.

8. **Dialyse supernatant extensively against Ni-NTA Lysis Buffer before continuing with the purification.**

9. **Proceed to protocols for *Strep*/6xHistidine-tag protein purification under native conditions (see protocols 8.1 to 8.2).**
   To allow an efficient *Strep*-tag/*Strep*-Tactin binding we strongly recommend using column purification instead of batch applications for proteins fused to Strep-tag II. It is crucial that protein binding takes place on the column. Even a pre-incubation of resin and lysate prior to filling the resin into a column will lead to decreased protein yields. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. If batch purification with the Strep-tag system is attempted, the use of Strep-Tactin Superflow High Capacity in combination with the One-STrEP-tag is recommended.

5 Detection of *Strep*-tag fusion proteins

5.1 Chemiluminescence detection of *Strep*-tag proteins with *Strep*-Tactin horse radish peroxidase (HRP) 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
- Enzyme dilution buffer: PBS with 0.2% BSA and 0.1 % Tween
- PBS-Tween buffer: PBS buffer with 0.1 % v/v Tween 20
- *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)
- Chemiluminescence detection solution

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).**
   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 *Strep*-tag proteins (step 5) add 10 µl Biotin Blocking Buffer (10 minutes, room temperature, gentle shaking).**
   This blocks endogenously biotinylated proteins (e.g. the biotin carboxyl carrier protein (BCCP, 22 kDa) in case of *E. coli*) which will otherwise stain sensitively.

5. **Pre-dilute *Strep*-Tactin horse radish peroxidase conjugate 1:100 in Enzyme dilution buffer (PBS, BSA, Tween) and add 10 µl to 10 ml PBS-Tween. 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).**

Develop chemoluminescence reaction according to the instruction of your respective kit.

5.2 Chemiluminescence detection of *Strep*-tag proteins with *Strep*MAB-Classic horse radish peroxidase (HRP) 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
- Enzyme dilution buffer: PBS with 0.2% BSA and 0.1 % Tween
- *Strep*-tag protein ladder can be used as positive control (cat. no. 2-1011-100)
- Chemiluminescence detection solution

1. **After SDS-PAGE and electrotransfer of the protein to an appropriate membrane block the membrane with 20 ml PBS-blocking buffer (PBS buffer with 3 % BSA and 0.5 % v/v Tween 20). Incubate: 1 h (room temperature; with gentle shaking) or overnight (4°C).**
   If you want to use milk powder for blocking, we recommend 1 % milk powder in PBS with 0.1 % Tween. Optimal dilution of *Strep*MAB-Classic horse radish peroxidase conjugate should then be 1:4000 (instead of 1:30000).
   We recommend the use of 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. **Pre-dilute *Strep*MAB-Classic horse radish peroxidase conjugate 1:10 in Enzyme dilution buffer (PBS, 0.2% BSA, 0.1% Tween) and add 3 µl to 10 ml PBS-Tween (when blocking with 1% milk powder in PBS-Tween use 4 µl undiluted *Strep*MAB-Classic horse radish peroxidase conjugate instead). Incubate 60 minutes at room temperature, gentle shaking.**

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

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

Develop chemiluminescence reaction according to the instruction of your respective kit.

5.3 Chromogenic detection of *Strep*-tag 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 DMF
- *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 transfer the proteins to an appropriate membrane.** We recommend using 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. **Optional: Before detection *Strep*-tag proteins (step 6) add 10 µl Biotin Blocking Buffer (10 minutes, room temperature, gentle shaking).** This blocks endogenously biotinylated proteins (e.g. the biotin carboxyl carrier protein (BCCP, 22 kDa) in case of *E. coli*) which will otherwise stain sensitively.

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.**

5.4 Chromogenic detection of *Strep*-tag proteins with *Strep*-Tactin horse radish peroxidase (HRP) 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
- 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 transfer of the proteins to an appropriate membrane, block the membrane with 20 ml PBS-blocking buffer. Incubate: 1 h (room temperature; gentle shaking) or overnight (4°C).**
   We recommend using 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 *Strep*-tag proteins (step 5) add 10 µl Biotin Blocking Buffer (10 minutes, room temperature, gentle shaking).**
   This blocks endogenously biotinylated proteins (e.g. the biotin carboxyl carrier protein (BCCP, 22 kDa) in case of *E. coli*) which will otherwise stain sensitively.

5. **Add 2.5 µl *Strep*-Tactin 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. **Proceed the chromogenic reaction under shaking until optimal signal:background ratio is achieved.**

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

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

5.5 Chromogenic detection of *Strep*-tag proteins with *Strep*MAB-Classic HRP 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
- 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 perform electro transfer 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 2.5 µl *Strep*MAB-Classic HRP conjugate.**

6. **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 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.**

5.6 Trouble shooting – Detection

5.6.1 No signal

The primary antibody and the secondary antibody are not compatible.	Use secondary antibody that was raised against the species in which the primary was raised (e.g primary is raised in rabbit, use anti-rabbit secondary).
Not enough primary or secondary antibody is bound to the protein of interest.	Use more concentrated antibody. Incubate longer (e.g. overnight) at 4°C.
Cross-reaction between blocking agent and primary or secondary antibody.	Use a mild detergent such as Tween 20 or switch blocking reagent (i.e. commonly used blocking reagents are milk, BSA, serum or gelatin).
Insufficient antigen.	Load at least an aggregate of 20-30 μg protein per lane; Use protease inhibitors; Run the recommended positive control.
The protein of interest is not abundantly present in the tissue.	Use an enrichment step to maximize the signal (e.g. prepare nuclear lysates for a nuclear protein, etc.).
Poor transfer of protein to membrane.	Check the transfer with a reversible stain such as Ponceau S; check that the transfer was not performed the wrong way; if using PVDF membrane make sure you pre-soak the membrane in MeOH then in transfer buffer.
Excessive washing of the membrane.	Do not over wash the membrane.
Too much blocking does not allow you to visualize your protein of interest.	Switch blocking reagents or block for less time, we recommend 3% BSA and 0.5 % v/v Tween 20 in PBS for 60 min.
Over-use of the primary antibody.	Use fresh antibody as the effective concentration is lowered upon each re-use.
Secondary antibody inhibited by sodium azide.	Do not use sodium azide together with HRP-conjugated antibodies.
Detection kit is old and substrate is inactive	Use fresh substrate.

5.6.2 High background

Blocking of non-specific binding might be absent or insufficient.	Increase the blocking incubation period and consider changing blocking agent. We recommend 3% BSA and 0.5 % v/v Tween 20 in PBS for 60 min. These can be included in the antibody buffers as well. **An alternative protocol can be provided, please inquire.**
The primary antibody concentration may be too high.	Titrate the antibody to the optimal concentration, incubate for longer but in more dilute antibody.
Incubation temperature may be too high.	Incubate blot at 4°C.
The secondary antibody may be binding non-specifically or reacting with the blocking reagent.	Run a secondary control without primary antibody.
Cross-reaction between blocking agent and primary or secondary.	Add a mild detergent such as Tween-20 to the incubation and washing buffer.
Washing of unbound antibodies may be insufficient.	Increase the number of washes.
Your choice of membrane may give high background.	Nitrocellulose membrane is considered to give less background than PVDF.
The membrane has dried out.	Care should be taken to prevent the membrane from drying out during incubation.

5.6.3 Multiple bands

Cell lines that have been frequently passaged gradually accumulate differences in their protein expression profiles.	Go back to the original non-passaged cell line and run the current and original cell line samples in parallel.
The protein sample has multiple modified forms in vivo such as acetylation, methylation, myristylation, phosphorylation, glycosylation etc.	Examine the literature and use an agent to dephosphorylate, de-glycosylate, etc. the protein to bring it to the correct size.

The target in your protein sample has been digested (more likely if the bands are of lower molecular weight).	Make sure that you incorporate sufficient protease inhibitors in your sample buffer.
Primary antibody concentration is too high - at high concentration multiple bands are often seen.	Try decreasing the antibody concentration and/or the incubation period.
Secondary antibody concentration is too high - at high concentration secondaries will bind nonspecifically.	Try decreasing the concentration. Run a secondary antibody control (without the primary).
The bands may be non-specific.	Where possible use blocking peptides to differentiate between specific and non-specific bands. Only specific bands should be blocked (and thus disappear).
The protein target may form multimers.	Try boiling in SDS-Page for 10 minutes rather than 5 minutes to disrupt multimers.

5.6.4 Uneven white "spots"on the blot

Air bubbles were trapped against the membrane during transfer or the antibody is not evenly spread on the membrane.	Make sure you remove bubbles when preparing the gel for transfer. Incubate antibodies under agitation.

5.6.5 Other Problems

Black dots on the blot

The antibodies are binding to the blocking agent.	Filter the blocking agent.

White bands on a black blot (negative of expected blot)

Too much primary and/or too much secondary antibody.	Dilute the antibodies more.

MW marker lane is black

The antibody is reacting with the MW marker.	Add a blank lane between the MW marker and the first sample lane.

6 Purification of *Strep*-tag fusion proteins

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

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 as well as *Strep*-Tactin Superflow high capacity can be used for gravity flow purification
- Binding capacity is protein dependent and normally lies between 50 and 100 nmol (up to 500 nmol in case of *Strep*-Tactin Superflow High Capacity) recombinant protein per ml bed volume (100 nmol correspond to 2 mg of a 20 kDa protein)
- 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. When using *Strep*-Tactin Superflow High Capacity resin it may be advantageous to use 5-10 mM desthiobiotin to get the target protein eluted at higher concentration.
- 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 6.
- 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.**
   Remove first top cap from column, then the cap at the outlet of the column. If the caps are removed in reverse order, the column may run dry. Remove storage buffer prior to adding Buffer W for equilibration.
   The column cannot run dry under gravity flow.
   Use buffer without EDTA for metalloproteins.

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

3. **Add supernatant of cleared lysates to the column.**
   The volume of the lysates should be in the range of between 0.5 and 10 CVs (see Table 4 on page 38). 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 (up to 500 nmol in case of *Strep*-Tactin Superflow high capacity) recombinant *Strep*-tag II fusion protein per 1 ml CV.

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

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

6.2 Purification of *Strep*-tag fusion proteins on chromatography workstations using H-PR cartridges

Material and important notes

- H-PR cartridges filled with 1 ml or 5 ml *Strep*-Tactin Superflow (High Capacity) or MacroPrep are designed for use with chromatography workstations with 10-32 fittings (HPLC and Äkta). They can, however, also be operated with other workstations, with syringes or with peristaltic pumps by use of appropriate adapter sets (Catalogue numbers 2-1021-001 (Luer lock), 2-1022-001(M6), 2-1023-001 (1/4-28), 2-1025-001 (1/16 inch); see www.iba-go.com/prottools/prot_fr01.html)
- CV = column bed volume
- Binding capacity is protein dependent and normally lies between 50 and 100 nmol (up to 500 nmol in case of *Strep*-Tactin Superflow High Capacity) recombinant protein per ml bed volume (100 nmol of a 20 kDa protein correspond to 2 mg)

- Cartridges may be connected in series to enlarge capacity. A coupling adapter is needed in this case (Cat. no. 2-1026-001)
- Recommended flow rates: 0.5-1 ml/min for a 1 ml cartridge; 1-3 ml/min for a 5 ml cartridge.
- 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. When using *Strep*-Tactin Superflow High Capacity resin it may be advantageous to use 5-10 mM desthiobiotin to get the target protein eluted at higher concentration.
- 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 6.
- Generally, it is recommended to perform chromatography at 4°C. Depending on the individual equipment this is not always possible and chromatography has to be performed at room temperature. If cartridges are stored at 4°C and are transferred to room temperature air bubbles may form due to restricted solubility of air at elevated temperatures. Therefore, it is recommended to equilibrate the cartridges immediately after exposure to higher temperatures with buffer that is equilibrated at such temperatures.
- Cartridges do not generate significant back pressure which makes the use of flow restrictors superfluous. Therefore, IBA recommends not to use flow restrictors to avoid inhomogeneities resulting from buffer changes during chromatography.

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

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

3. **Apply lysate to H-PR cartridge.**
   Begin with a flow rate of 1 ml/min. Monitor pressure at this step. If the lysate is very viscous and pressure is increased significantly, reduce viscosity of the extract (please note Table 4 in section 6.1 on page 38 in this respect) or reduce flow rate.
   Collect the flow-through for SDS-PAGE analysis.

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

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

6.3 Purification of *Strep*-tag fusion proteins using H-PR cartridges with syringes

Material and 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).
- 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. When using *Strep*-Tactin Superflow High Capacity resin it may be advantageous to use 5-10 mM desthiobiotin to get the target protein eluted at higher concentration.
- 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 6.
- Generally, it is recommended to perform chromatography at 4°C. Depending on the individual equipment this is not always possible and chromatography has to be performed at room temperature. If cartridges are stored at 4°C and transferred to room temperature air bubbles may form since cold storage buffer is able to take up more gas than buffers at ambient temperature. Therefore, it is recommended to equilibrate the cartridges immediately after exposure to higher temperatures with buffer that is equilibrated at such temperatures.
- Cartridges may be connected in series to enlarge capacity. A coupling adapter is necessary in this case (Cat. no. 2-1026-001).

Protocol for running a 1 ml H-PR cartridge.

1. **Connect the adapters and fill the inlet with Buffer W.**

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

3. **Inject 5 ml Buffer W with a flow rate of 1 drop/sec to equilibrate the H-PR cartridge**

4. **Centrifuge the cleared lysate (14,000 rpm, 5 minutes, 4°C, microfuge) to remove aggregates that may have formed during storage.**
   Insoluble aggregates which may clog the cartridge shall be removed.

5. **Fill a syringe with the appropriate amount of the cleared lysate.**

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

7. **Fill the inlet with Buffer W.**

8. **Apply the cleared lysate with a flow rate of 0.3 to 0.5 drops/sec.**

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

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

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

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

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

6.4 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 on page 6.
- 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.
- Cartridges do not generate significant back pressure which makes the use of flow restrictors superfluous. Therefore, IBA recommends not to use flow restrictors to avoid inhomogeneities resulting from buffer changes during chromatography.

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.

6.5 Trouble shooting – *Strep*-tag purification

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

pH is not correct.	The pH should be > 7.0
Strep-tag II is not present.	Use protease deficient *E. coli* expression strains. 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. *Avoid purification in discontinuous batch mode*. Prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag.
Strep-tag II is partially accessible.	Reduce washing volume to 3 CVs.
Strep-Tactin column is inactive.	Check activity with HABA. Add avidin (Biotin Blocking Buffer) if biotin containing extracts are intended to be purified. The total biotin content of the soluble part of the total E. coli cell lysate is about 1 nmol per liter culture (OD_{550} = 1.0). Add 2-3 nmol of avidin monomer per nmol of biotin.
Batch purification is carried out	To allow an efficient *Strep*-tag/*Strep*-Tactin binding we strongly recommend using column purification instead of batch applications. It is crucial that protein binding takes place on the column. Even a pre-incubation of resin and lysate prior to filling the resin into a column will lead to decreased protein yields. If batch purification with the Strep-tag system is intended, the use of Strep-Tactin Superflow High Capacity in combination with the One-STrEP-tag is recommended.
Flow rate is too fast	Reduced flow rates may increase yields depending on the given recombinant protein.

6.5.2 "Contaminating proteins"

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

Contaminants are short forms of the tagged protein.	Use protease deficient *E. coli* expression strains. Add protease inhibitors after cell lysis. Fuse the *Strep*-tag II with the other protein terminus. Check for the presence of internal translation initiation starts (only in case of C- terminal *Strep*-tag II) or premature termination sites (only in case of N-terminal *Strep*-tag II). Add 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).

6.5.3 "Air bubbles in the column"

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

To prevent 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 6xHistidine-tag proteins

7.1 Batch purification of 6xHistidine-tag proteins under native conditions

Material and important notes

- The amount of cells required depends on the expression level of the 6xHistidine-tag protein and the expression system used.
- The binding capacity of Ni-NTA resins is protein dependent and normally lies between 5 and 10 mg/ml (e.g Ni-NTA Sepharose or Superflow 300-500 nmol of a 20 kDa protein).
- The resins are supplied as 50% slurries.
- 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, 5-10 mM β-ME, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information see Table 2 on page 7.

1. **Add 1 ml of the 50% Ni-NTA slurry to 4 ml cleared lysate (in Ni-NTA Lysis Buffer; see protocol 4.3 or 4.4) and mix gently by shaking (200 rpm on a rotary shaker) at 4°C for 60 min.**
   10 mM (or 20mM) imidazole in Ni-NTA Lysis Buffer suppresses the binding of non-tagged contaminating proteins and leads to greater purity after fewer washing steps. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1-5 mM.

2. **Load the lysate/Ni-NTA mixture onto a column with capped bottom outlet.**
   Empty columns may be purchased e.g. from BioRad, Munich.

3. **Remove bottom cap and collect the column flow-through.**
   Save flow-through for SDS-PAGE analysis.

4. **Wash twice with 4 ml Ni-NTA Wash Buffer; collect wash fractions for SDS-PAGE analysis.**
   20 mM imidazole in the Ni-NTA Wash Buffer elutes non-tagged contaminating proteins. Imidazole concentration can be reduced to 1-5 mM imidazole. This can result in loss of purity, but higher yields of recombinant protein.

5. **Elute the protein 4 times with 0.5 ml Ni-NTA Elution Buffer.**
   Collect the eluate in four tubes and analyse by SDS-PAGE.

7.2 Gravity flow purification of 6xHistidine-tag proteins under native conditions

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 lysis, wash and elution buffers can be modified to suit the particular apllication, 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 2 on page 7.
- 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.

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 cleared lysate from protocol 4.3 or 4.4 (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. **Once the cell extract has completely entered the column, wash the column 4 times with 2 CV Ni-NTA Wash Buffer.**
   Collect the eluate in 2 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.

7.3 Purification of 6xHistidine-tag fusion proteins under native conditions on chromatography workstations using Ni-NTA Superflow cartridges H-PR

Material and important notes

- H-PR cartridges 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-go.com/prottools/prot_fr01.html)
- CV = column bed volume
- 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).
- Recommended flow rates:
  0.5-1 ml/min for 1 ml cartridges; 1-3 ml/min for 5 ml cartridges
- Ni-NTA Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 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, 5-10 mM β-mercaptoethanol, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information see Table 2 on page 7.
- Cartridges do not generate significant back pressure which makes the use of flow restrictors superfluous. Therefore, IBA recommends not to use flow restrictors to avoid inhomogeneities resulting from buffer changes during chromatography.

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

2. **Equilibrate H-PR cartridge with 5 CVs of Ni-NTA Lysis Buffer.**
   The flow rate should be between 0.5 and 1 ml/minute for 1 ml cartridges and between 1 and 3 ml/minute for 5 ml cartridges.
   Monitor elution at 280 nm; the baseline should be stable after washing with 5 CVs.

3. **Apply lysate (see protocol 4.3 or 4.4) to H-PR cartridge and wash with Ni-NTA Lysis Buffer until A_{280} is stable.**
   Usually 5-10 CVs are sufficient. Begin with a flow rate of 1 ml/min. Monitor pressure at this step. If the lysate is very viscous, reduce flow rate or viscosity of the lysate.
   Collect the flow-through for SDS-PAGE analysis.

4. **Wash with Ni-NTA Wash Buffer until A_{280} is stable.**
   Usually 5-10 CVs are sufficient.
   Collect fractions for SDS-PAGE analysis.

5. **Elute the protein with Ni-NTA Elution Buffer.**
   In some cases a step-gradient of Ni-NTA Elution Buffer in Ni-NTA Wash Buffer may be used to elute the protein. Five CVs at each step are usually sufficient. The 6xHistidine-tag protein usually elutes in the second and third CV.
   Imidazole absorbs at 280 nm, which should be considered when monitoring protein elution. If small amounts of 6xHistidine-tag proteins are purified, elution peaks may be poorly visible.

7.4 Purification of 6xHistidine-tag fusion proteins under native conditions using Ni-NTA Superflow cartridges H-PR with syringes

Material and 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 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, 5-10 mM β-ME, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information see Table 2 on page 7.
- 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.

Protocol for a 1 ml H-PR cartridge.

1. **Fill the 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 5 ml Ni-NTA Lysis Buffer with a flow rate of 1 drop/sec to equilibrate the cartridge H-PR.**

4. **Centrifuge the cleared lysate from protocol 4.3 or 4.4 (14,000 rpm, 5 minutes, 4°C, microfuge) to remove aggregates that may have formed during storage.**

5. **Fill a syringe with the appropriate amount of the cleared lysate.**

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

7. **Fill the H-PR cartridge inlet with Ni-NTA Lysis Buffer and connect the syringe containing the cleared lysate.**

8. **Apply the cleared lysate with a flow rate of 0.3 to 0.5 drops/sec.**

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 H-PR cartridge.**

10. **Wash the H-PR cartridge with 160 drops (corresponding to approx. 8 ml) Ni-NTA Wash Buffer at a flow rate of 0.3 to 0.5 drops/sec. Collect the eluate in fractions of 20 drops and apply 2 µl of the first fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE (fraction W1 to W8).**

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

12. **Fill a syringe with 4 ml Ni-NTA Elution Buffer and connect to the H-PR cartridge.**

13. **Elute the recombinant 6xHistidine-tag fusion protein with 60 drops Ni-NTA Elution Buffer at a flow rate of 0.3 to 0.5 drops/sec. Collect the eluate in fractions of 10 drops and apply 20 µl of each fraction to an analytical SDS-PAGE (fraction E1-E6). Purified protein should be present in fraction E2-E5.**

7.5 Batch purification of 6xHistidine-tag proteins under denaturing conditions

Material and important notes

- The amount of cells required depends on the expression level of the 6xHistidine-tag protein and the expression system used.
- The binding capacity of Ni-NTA resins is protein dependent and normally lies between 5 and 10 mg/ml (e.g. 300-500 nmol of a 20 kDa protein).
- The resins are supplied as 50% slurries.
- Cells can be lysed in either 6 M GuHCl or 8 M urea. If GuHCl is used all buffers must include 6 M GuHCl instead of 8 M urea.
- Buffer B: 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 8
- Buffer C (Washing Buffer): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 6.3
- Buffer D1 (Elution Buffer1): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 5.9
- Buffer D2 (Elution Buffer2): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 4.5

Due to the dissociation of urea, the pH of urea containing buffers should be adjusted immediately prior to use. Do not autoclave.

1. **Add 1 ml of the 50% Ni-NTA slurry to 4 ml lysate (see protocol 4.5) and mix gently by shaking (e.g., 200 rpm on a rotary shaker) for 15-60 minutes at room temperature.**
   For proteins that are expressed at very high levels (50-100 mg of 6xHistidine-tag protein per liter of cell culture), a 5x concentrated cell lysate (resuspend the pellet from a 20 ml culture in 4 ml Buffer B) can be used. 4 ml of a 5x concentrated cell lysate in Buffer B will contain approximately 1-2 mg of 6xHistidine-tag protein. For much lower expression levels (1-5 mg/liter), 200 ml of cell culture should be used for a 50x concentrated cell lysate (4 ml cell lysate = 0.2-1 mg of 6xHistidine-tag protein).

2. **Load lysate-resin mixture carefully into an empty column with the bottom cap still attached.**

3. **Remove the bottom cap and collect the flow-through.**
   Collect flow-through for SDS-PAGE analysis.

4. **Wash twice with 4 ml Buffer C.**
   Keep wash fractions for SDS-PAGE analysis.

5. **Elute the recombinant protein 4 times with 0.5 ml Buffer D1, followed by 4 times with 0.5 ml Buffer D2.**
   Collect fractions and analyze by SDS-PAGE.

7.6 Gravity flow purification of 6xHistidine-tag proteins under denaturing conditions

Material and important notes

- **CV = column bed volume**
- Ni-NTA Superflow and Ni-NTA Sepharose are available for gravity flow column chromatography.
- 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).
- Cells can be lysed in either 6 M GuHCl or 8 M urea. If GuHCl is used all buffers must include 6 M GuHCl instead of 8 M urea.
- Buffer B: 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 8
- Buffer C (Washing Buffer): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 6.3
- Buffer D1 (Elution Buffer1): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 5.9
- Buffer D2 (Elution Buffer2): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 4.5

Due to the dissociation of urea, the pH of urea containing buffers should be adjusted immediately prior to use. Do not autoclave.

1. **Equilibrate the Ni-NTA column with 2 CVs Buffer B.**
   Storage buffer is removed.
   The column cannot run dry under gravity flow.

2. **Centrifuge cleared lysate from protocol 4.5 (14,000 rpm, 5 minutes, 4°C, microfuge).**
   Insoluble aggregates which can clog the column are removed.

3. **Add cleared lysate supernatant to the column.**

4. **After the cell extract has completely entered the column, wash the column 8 times with Buffer C.**
   Collect the eluate in 1 CV fractions and apply 2 µl of the first fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE.

5. **Add 6 times 0.5 CVs Buffer D1 and collect the eluate in 0.5 CV fractions.**
   Samples of each fraction can be used for SDS-PAGE analysis.
   If elution is incomplete with Buffer D1, Buffer D2 should be used.

7.7 Purification of 6xHistidine-tag proteins under denaturing conditions on chromatography workstations using Ni-NTA Superflow cartridges H-PR

Material and important notes

- H-PR cartridges 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-go.com/prottools/prot_fr01.html)
- CV = column bed volume
- The amount of cells required depends on the expression level of the 6xHistidine-tag protein and the expression system used.
- The binding capacity of Ni-NTA resins is protein dependent and normally lies between 5 and 10 mg/ml (e.g 300-500 nmol of a 20 kDa protein).
- Cells can be lysed in either 6 M GuHCl or 8 M urea. If GuHCl is used all buffers must include 6 M GuHCl instead of 8 M urea.
- Buffer B: 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 8
- Buffer C (Washing Buffer): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 6.3
- Buffer D1 (Elution Buffer1): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 5.9
- Buffer D2 (Elution Buffer2): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 4.5

Due to the dissociation of urea, the pH of urea containing buffers should be adjusted immediately prior use. Do not autoclave.

- Cartridges do not generate significant back pressure which makes the use of flow restrictors superfluous. Therefore, IBA recommends not using flow restrictors to avoid inhomogeneities resulting from buffer changes during chromatography.

1. **Connect the Ni-NTA Superflow cartridge H-PR to the workstation.**

2. **Equilibrate H-PR cartridge with 5 CVs of Buffer B.**
   The recommended flow rate is 0.5-1 ml/min for 1 ml cartridges and 1-3 ml/min for 5 ml cartridges.
   Monitor elution at 280 nm; the baseline should be stable after washing with 5 CVs.

3. **Apply lysate (see protocol 4.5) to the H-PR cartridge and wash with Ni-NTA Lysis Buffer until A_{280} is below 0.01.**
   Usually 5-10 CVs are sufficient. 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 recommended value (25 psi). If necessary reduce flow rate.
   Collect the flow-through for SDS-PAGE analysis.

4. **Wash with Buffer C until A_{280} is below 0.01.**
   Usually 5-10 CVs are sufficient. Collect fractions for SDS-PAGE analysis.

5. **Elute the protein with Buffer D1.**
   If elution is incomplete with Buffer D1, Buffer D2 should be used.

7.8 Denaturing purification of 6xHistidine-tag fusion proteins using Ni-NTA Superflow cartridges H-PR with syringes

Material and 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)
- CV = column bed volume
- Ni-NTA Superflow cartridges are available for injection.
- 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).
- Maximal pressure 140 psi, bead size 60-160 µm
- Cleared lysate (E. coli) from a 20-200 ml culture (see protocol 4.5, page 27)
- Cells can be lysed in either 6 M GuHCl or 8 M urea. If GuHCl is used all buffers must include 6 M GuHCl instead of 8 M urea.
- Buffer B: 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 8
- Buffer C (Washing Buffer): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 6.3
- Buffer D1 (Elution Buffer1): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 5.9
- Buffer D2 (Elution Buffer2): 100 mM NaH_2PO_4, 10 mM Tris·HCl, 8 M urea, pH 4.5

Due to the dissociation of urea, the pH of urea containing buffers should be adjusted immediately prior to use. Do not autoclave.

Protocol for a 1 ml cartridge H-PR.

1. **Fill the inlet with Buffer B. Inject 5 ml Buffer B (flow rate 1 drop/sec). Avoid the inclusion of air bubbles.**

2. **Centrifuge cleared lysate from protocol 4.5 to remove insoluble aggregates (14,000 rpm, 5 minutes, 4°C, microfuge).**

3. **Fill the inlet with Buffer B.**

4. **Fill a syringe with the supernatant from step 2. Inject the supernatant with a flow rate of 0.3 to 0.5 drops/sec.**

5. **Fill the inlet with Buffer C.**

6. **Wash with 160 drops (approx. 8 ml) Ni-NTA Wash Buffer. Collect the eluate 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).**

7. **Fill the inlet with Buffer D1.**

8. **Fill a 5 ml injection with 3.5 ml Buffer D1.**
   If elution is incomplete with Buffer D1, Buffer D2 should be used.

9. **Inject the Buffer D1 with a flow rate of 0.3 to 0.5 drops/sec.**

10. **Elute with 60 drops Buffer D1. Collect the elution of 10 drops and apply a sample of each fraction to an analytical SDS-PAGE (fraction E1 to E6).**

7.9 Trouble shooting – 6xHistidine-tag purification

7.9.1 "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.	Purify protein under denaturing conditions. 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. Dissociation of urea often causes a shift in pH. 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.

7.9.2 "Protein elutes in 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.

7.9.3 "Protein precipitates during purification"

Temperature is too low.	Perform purification at room temperature.
Protein forms aggregates.	Try adding solubization 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.

7.9.4 "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.

7.9.5 "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.

7.9.6 "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 Purification of *Strep*/6xHis-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 or the One-STrEP-tag 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 or One-STrEP-tag epitope) is loaded directly onto a *Strep*-Tactin matrix, preferentially *Strep*-Tactin Superflow High Capacity. 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 results in a protein preparation of higher concentration but renders the column inactive thus preventing its re-use.

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

8.1.1 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, 5-10 mM β-mercaptoethanol, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information see Table 1 on page 6.
- 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 cleared lysate from protocol 4.6 or 4.7 (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 extract 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.**

8.1.2 Second step: *Strep*-Tactin chromatography

Material and important notes

- **CV = column bed volume**
- *Strep*-Tactin Sepharose, Superflow and MacroPrep, preferentially *Strep*-Tactin Superflow High Capacity, can be used for gravity flow purification.
- Binding capacity is protein dependent and normally lies between 50 and 100 nmol (up to 500 nmol in case of *Strep*-Tactin Superflow High Capacity) recombinant protein per ml bed volume (100 nmol correspond to 2 mg of a 20 kDa protein)
- 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. When using *Strep*-Tactin Superflow High Capacity resin it may be advantageous to use 5-10 mM desthiobiotin to get the target protein eluted at higher concentration.
- 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 6.
- 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.

8.2 Purification of *Strep*/6xHistidine-tag fusion proteins under native conditions using H-PR cartridges with syringes

8.2.1 First step: Ni-NTA chromatography

Material and 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)
- 1 ml and 5 ml Ni-NTA Superflow cartridges H-PR are available and can be run under pressure using syringes or chromatography workstations.
- 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, 5-10 mM β-mercaptoethanol, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information see Table 1 on page 6.
- 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.

8.2.2 Second step: *Strep*-Tactin chromatography

Material and important notes

- 1 ml and 5 ml *Strep*-Tactin Superflow (optionally with high capacity) or MacroPrep cartridges H-PR are available and can be run using syringes or chromatography workstations.
- Binding capacity is protein dependent and normally lies between 50 and 100 nmol (up to 500 nmol in case of *Strep*-Tactin Superflow High Capacity) recombinant protein per ml bed volume (100 nmol of a 20 kDa protein correspond to 2 mg). As it is inferior (except *Strep*-Tactin Superflow High Capacity) to the Ni-NTA binding capacity, care should be taken to avoid overloading of the *Strep*-Tactin cartridge H-PR resulting in loss of protein. For yield optimization, a 5 ml *Strep*-Tactin (normal capacity) or a 1 ml *Strep*-Tactin Superflow High Capacity cartridge H-PR may be combined with a 1 ml Ni-NTA cartridge H-PR.
- 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 6.
- 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.
- H-PR cartridges may be connected for direct loading of the *Strep*-Tactin cartridge H-PR with the eluate of the Ni-NTA cartridge H-PR. A coupling adapter is necessary in this case (Cat. no. 2-1026-001).

Protocol for the purification of a *Strep*/Histidine fusion protein by the sequential use of 1 ml cartridges H-PR and syringes. Alternatively, purification may be performed on chromatography workstations by combining protocols described under 7.3 and 6.2.

1. **Connect the luer adapter to the cartridge H-PR inlet and the 1/16 inch adapter to the outlet.**

2. **Fill the inlet with Ni-NTA Lysis Buffer.**

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

4. **Inject 5 ml Ni-NTA Lysis Buffer with a flow rate of 1 drop/sec to equilibrate the H-PR cartridge.**

5. **Centrifuge the cleared lysate from protocol 4.6 or 4.7 (14,000 rpm, 5 minutes, 4°C, microfuge) containing the *Strep*/6xHistidine-tag fusion protein to remove aggregates that may have formed during storage.**

6. **Fill a syringe with the appropriate amount of the cleared lysate.**

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

8. **Fill the inlet with Ni-NTA Lysis Buffer and connect the syringe containing the cleared lysate.**

9. **Apply the cleared lysate with a flow rate of 0.3 to 0.5 drops/sec.**

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

11. **Wash the H-PR cartridge with 160 drops (corresponding to approx. 8 ml) Ni-NTA Wash Buffer at a flow rate of 0.5 drops/sec. Collect the eluate in fractions of 20 drops and apply 2 µl of the first fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE (fraction W1 to W8).**

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

13. **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 nickel will be stripped off the NTA matrix which must be regenerated prior to re-use for purification of a 6xHistidine-tag protein (see protocol 9.2.1).

14. **Fill the inlet of a *Strep*-Tactin resin cartridge H-PR with Ni-NTA Elution Buffer.**

15. **Connect the Ni-NTA cartridge H-PR with the *Strep*-Tactin cartridge H-PR by using a coupling adapter.**

16. **Inject Ni-NTA Elution Buffer with a flow rate of 0.3 to 0.5 drops/sec and collect the flow through and apply 20 µl to an analytical SDS-PAGE.**
    The *Strep*/6xHistidine-tag fusion protein will elute from the Ni-NTA and bind to the *Strep*-Tactin resin.

17. **Disconnect the Ni-NTA cartridge H-PR from the *Strep*-Tactin cartridge H-PR and connect a luer adapter to the *Strep*-Tactin cartridge H-PR inlet.**

18. **Fill the inlet of the *Strep*-Tactin cartridge H-PR with Buffer W.**

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

20. **Wash with 100 drops Buffer W at a flow rate of 0.3 to 0.5 drops/sec. Collect the eluate in fractions of 20 drops and apply 20 µl of each fraction to an analytical SDS-PAGE (fraction W6 to W10).**

21. **Fill the inlet with Buffer E.**

22. **Fill a syringe with 4 ml Buffer E and inject Buffer E with a flow rate of 0.3 to 0.5 drops/sec.**

23. **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.**

9 APPENDIX

9.1 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 recommended 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.
- HABA cannot be efficiently removed from Strep-Tactin Superflow High Capacity by using Buffer W. We recommend to use Buffer W at pH 10.5 (or alternatively 100 mM Tris base) for efficient removal of HABA from Strep-Tactin Superflow High Capacity.

9.1.1 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. Exception: In case of Strep-Tactin Superflow High Capacity, use 4 CV Buffer W at pH 10.5 for HABA removal. Immediately afterwards, exchange the column buffer to Buffer W pH 8.0 as long term exposure to pH 10.5 may be detrimental to the resin.**

9.1.2 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. Exception: In case of Strep-Tactin Superflow High Capacity, use 4 CV Buffer W at pH 10.5 for HABA removal. Immediately afterwards, exchange the column buffer to Buffer W pH 8.0 as long term exposure to pH 10.5 may be detrimental to the resin.**

9.2 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.

9.2.1 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.**

10 References

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

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