

# **One-STrEP™ Kit**

## **One-STrEP-tag Purification for the Isolation and Identification of Protein Complexes in Mammalian Cells**

**A comprehensive manual**

Last date of revision  
May 2008

Version PR13-0011

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# 1 Introduction

## 1.1 One-STrEP-tag in protein:protein-interaction analysis

Protein:protein-interactions (PPI) are of central importance for virtually every process in a living cell. To understand the principles underlying the concerted action of proteins in time and space to generate cellular functions is a formidable challenge in modern biological science. Proteins might interact for a long time to form a protein complex, a protein may be carrying another protein (for example, from cytoplasm to nucleus or vice versa in the case of the nuclear pore importins), or a protein may interact briefly with another protein just to modify it (for example, a protein kinase will add a phosphate to a target protein). This modification of proteins itself can change PPI. For example, some proteins with SH2 domains only bind to other proteins when they are phosphorylated. Thus the “interactome” is realized to be a very dynamic network of protein complexes with changing compositions strictly depending on the given physiological state.

Initial approaches to study protein:protein-interactions were based on *ex vivo* and *in vitro* systems such as two hybrid systems and pull down assays which needed to be integrated with more physiological approaches to assess the complexity of PPI in a living cell. In 1999, Rigaut and co-workers developed a generic procedure to co-purify proteins expressed at their natural level with a TAP-tagged bait protein under native conditions using tandem affinity purification [1; cf. Figure 1]. This technology enabled in 2002 the *in vivo* elucidation of protein complexes in yeast in a large scale format [2]. However, consisting of a protein A domain and calmodulin binding peptide separated by a TEV protease cleavage site, the TAP tag features a size of 21 kDa and requires extended washing and time consuming cleavage steps making it prone to artefacts and incapable of detecting transient interaction partners [3].

In 2005, the 3 kDa sized One-STrEP-tag has been shown to enable a rapid one-step purification of protein complexes on Strep-Tactin Superflow at a purity comparable to the outcome of TAP technology which, in contrast, needs 2 sequential affinity purification steps [4]. An intact PP2A holoenzyme protein complex was purified from a cultured mammalian cancer cell line. The subsequent analysis of the complex by mass spectrometry identified, as expected, the known interaction partners and, remarkably, also a novel transient partner being a substrate of PP2A. Thus, the simple and rapid One-STrEP-tag procedure was proven in that example to be even an improvement over TAP for the isolation of transient interaction partners. The usefulness of the One-STrEP-tag approach for the isolation of functional protein complexes was further validated in many other systems including i) the purification of ternary complexes consisting of mRNA, ribosome and nascent polypeptides [5,6], ii) the co-purification of a putative replicative helicase (MCM2-7) and histones H3 and H4 together with the Strep-tagged histone chaperone Asf1 contributing to elucidation of nucleosome disruption and reassembly during DNA replication [7] or iii) the co-purification of 15 subsequently identified interacting proteins involved in cell migration with IKAP-Strep (bait, I $\kappa$ B-kinase associated protein, 150 kDa) [8]. Thus, one-step isolation of protein complexes by One-STrEP-tag for further analysis is a useful starting point and corresponding methods and reagents are provided by this kit.

Owing to the complexity of the field dealing with elucidation of protein:protein-interactions, no silver bullet can be provided with the current kit so far and alternative approaches should also be mentioned in this context. For example, despite the aforementioned results, demonstrating that the fast One-STrEP-tag purification makes even weakly associated protein complexes amenable to functional studies or mass spectroscopic identification, others adhere to the idea of the need for a further purification step involving a second tag with antibody affinity to obtain the protein complex in sufficient purity [3]. To meet the eventual need for a second purification step in certain formats, IBA has made the following development: A monoclonal antibody, named StrepMAB-Classic, with reversible binding properties had been generated against One-STrEP-tag and immobilized to MacroPrep support. Bound One-STrEP-tagged fusion protein can be competitively displaced using 0.5 mM of free Strep-tag II peptide in a physiological buffer. Thus, this novel resin allows a second efficient and mild purification step, completely independent – with respect to affinity receptor and matrix - from the first step on Strep-Tactin Superflow, thereby enabling a TAP approach relying on one tag only. The advantage is keeping modifications of the bait minimal to avoid unphysiological effects thereby enabling analysis of authentic PPIs. Pre-packed gravity flow StrepMAB-Classic columns (Cat. Nos. 2-1526-001 (1x 1ml bed volume) and 2-1526-505 (5x 0.2ml bed volume)) as well as the synthetic Strep-tag II peptide for elution (Cat. No. 2-1018-002) are not included in the kit but available separately.

As already discussed in the introduction, the dynamic nature of the interactome involves changing embodiments of interacting proteins. Many physiological processes are very fast and could not be resolved by the currently comparatively slow approach with the result that the involved PPI may escape detection. Addressing this problem, a Strep-tag-protein interaction experiment (SPINE) had been developed which combines the advantages of highly specific purification of proteins carrying a Strep-tag with reversible formaldehyde crosslinking to obtain a time-resolved snapshot of *in vivo* protein interactions [9]. At a certain stage, the cell culture is treated with formaldehyde which rapidly enters the cells and immediately links proteins which are in close contact to each other (about 2 Å) making the snapshot amenable to further analysis. Please inquire at IBA for the latest products dealing with that technology.

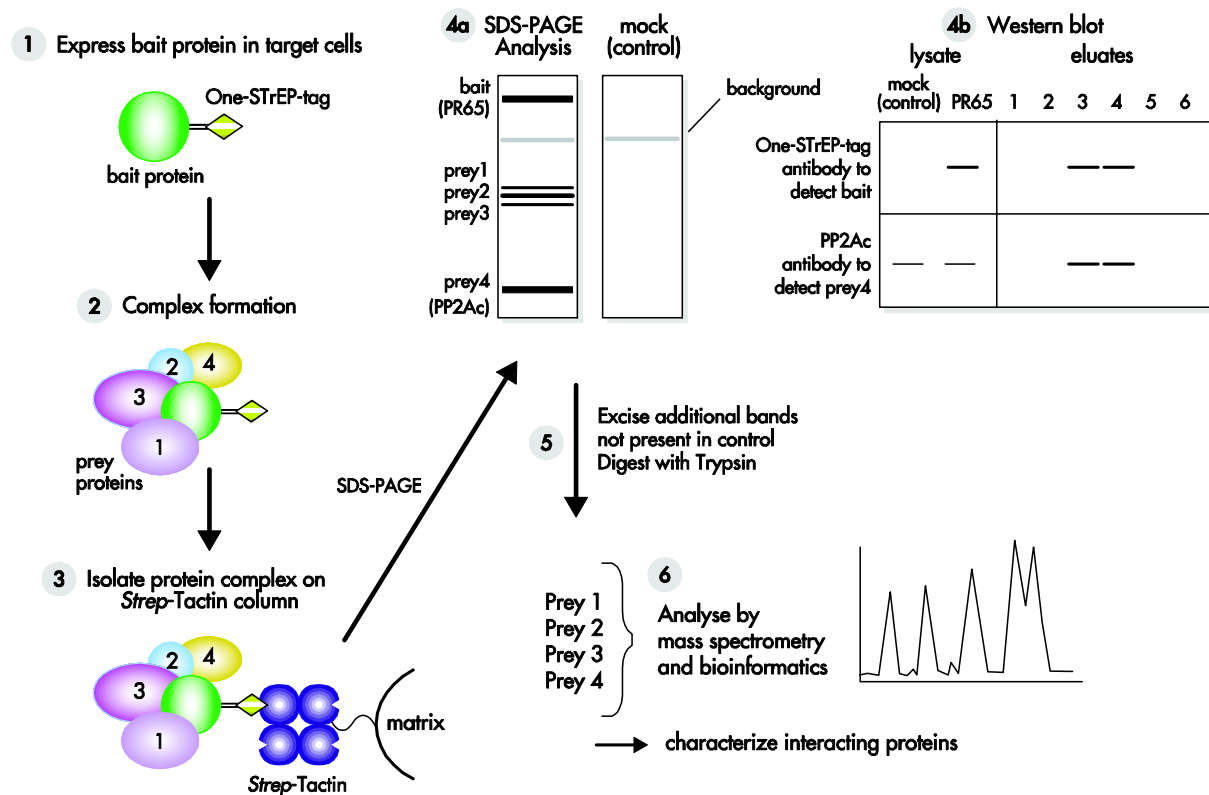
Concluding, use of Strep-tag technology with the novel One-STrEP-tag offers many solutions to meet the interactome elucidation challenge including:

- Fast one-step purification of weakly associated protein complexes
- One-tag TAP with Strep-Tactin Superflow and StrepMAB-Classic MacroPrep
- SPINE

IBA's high capacity cloning system StarGate simplifies the systematic screen for finding the optimal Strep-tag or One-STrEP-tag positioning on the bait, optionally also in combination with a further tag, and the optimal expression system. Please note that the vectors provided with this kit are stand alone and not compatible with StarGate. Further information on StarGate is available at [www.stargate-cloning.com](http://www.stargate-cloning.com).

Further high end tools around Strep-tag like the irreversibly binding monoclonal antibody StrepMAB-Immo complete the tool box for downstream analysis of protein complex

functions by offering directed and mild immobilization on, e.g., BiaCore chips or microplates [10].



**Figure 1: Schematic illustration of the entire process of identifying interacting proteins**

Cells are transfected with the plasmid coding for the bait protein fused to the One-STRrEP-tag. After an appropriate expression period (1,2), cells are lysed and the bait together with bound proteins are isolated using a Strep-Tactin column (3). Proteins are resolved on a SDS-gel (4a) and specifically bound proteins e.g. proteins not appearing in the mock control can be identified. This is done by either excising the protein bands from the gel and subsequent mass spectrometric analysis (5,6) or, if interaction partners are known or expected, by Western blot using specific antibodies (4b).

## 1.2 The One-STrEP-Kit

The Kit includes various *Strep*-Tactin columns and buffers for the isolation of protein complexes in different scales. For detection of the bait by means of immunoblotting, One-STrEP-tag specific *Strep*MAB-Classic conjugated to horse radish peroxidase is included. As a control for the entire procedure, a control plasmid encoding a One-STrEP-tag fusion protein as bait and antibodies against a specifically binding prey are included as well. The control plasmid encodes PR65 alpha, a scaffolding subunit of protein phosphatase 2A (PP2A), fused to the One-STrEP-tag at its C-terminus. Using the supplied *Strep*MAB-Classic HRP conjugate directed against the One-STrEP-tag, it can be used as positive control for transfection, expression, purification and detection in the cell line of choice. In addition, using the provided polyclonal antibodies against one prey of PR65 alpha, the catalytic subunit of PP2A, a positive control for the successful isolation of an intact protein complex (phosphatase PP2A holoenzyme) is possible as well [4].

## 1.3 pEXPR-IBA vectors 103 and 105

One-STrEP-tag vectors pEXPR-IBA 103 and 105 are designed for C- and N-terminal fusion of the One-STrEP-tag to the bait protein respectively. They provide high-level expression and purification of recombinant proteins in mammalian hosts. The human cytomegalovirus immediate-early (CMV) promoter provides strong expression in a wide range of mammalian cells. To prolong expression in transfected cells, the vector will replicate in cell lines that are latently infected with SV40 large T antigen (e.g. COS7). In addition, Neomycin resistance gene allows direct selection of stable cell lines.

Please note that the vectors provided with this kit are stand alone and not compatible with StarGate. IBA's high capacity cloning system StarGate simplifies the systematic screen for finding the optimal *Strep*-tag or One-STrEP-tag positioning on the bait, optionally also in combination with a further tag, and the optimal expression system. Further information on StarGate is available at [www.stargate-cloning.com](http://www.stargate-cloning.com).

## 1.4 One-STrEP-tag/*Strep*-Tactin<sup>®</sup> system

The One-STrEP-tag is a modification of our successful *Strep*-tag II. The tag has been adapted to higher detergent compatibility, which is important for optimizing binding and washing conditions for protein-protein interactions. This technology allows one-step purification of almost any recombinant protein under physiological conditions, thus preserving its bioactivity.

After application of the crude extract to a *Strep*-Tactin column and a short washing step, gentle but highly efficient elution of recombinant bait and all bound proteins is performed by addition of low concentrations of biotin as provided by Buffer BE (2 mM). Please note that re-use of the *Strep*-Tactin columns is not possible after elution with the most efficient competitor biotin. We include biotin as regeneration of the columns is not recommended

whatsoever because results may be jeopardised through cross-contaminations. If, however, preliminary purifications are intended to optimize conditions, elution should be performed by using Buffer E (Cat. No. 2-1000-025; not provided within this kit) including desthiobiotin, which allows regeneration of the columns but provides less efficient elution in comparison to biotin.

The One-STrEP-tag/*Strep*-Tactin interaction is compatible with a variety of reagents (see Table 1 but consult also [www.iba-go.com/downloads](http://www.iba-go.com/downloads) for latest updates). Comprehensive reviews and scientific publications giving an overview of various *Strep*-tag applications are listed at [www.iba-go.com](http://www.iba-go.com).

Reagent	Concentration
<b>Reduction Agents</b>	
DTT	50 mM
$\beta$ -mercaptoethanol	50 mM
<b>Non-ionic Detergents</b>	
C <sub>8</sub> E <sub>4</sub> Octyltetraoxyethylene	max. 0.88 %
C <sub>10</sub> E <sub>5</sub> ; Decylpentaoxyethylene	0.12 %
C <sub>10</sub> E <sub>6</sub>	0.03 %
C <sub>12</sub> E <sub>8</sub>	0.005 %
C <sub>12</sub> E <sub>9</sub> ; Dodecyl nonaoxyethylene (Thesit)	0.023 %
DM; Decyl- $\beta$ -D-maltoside	0.35 %
LM; N-dodecyl- $\beta$ -D-maltoside	0.007 %
NG; N-nonyl- $\beta$ -D-glucopyranoside	0.2 %
OG; N-octyl- $\beta$ -D-glucopyranoside	2.34 %
TX; Triton X-100	2 %
Tween 20	2 %
<b>Ionic Detergents</b>	
N-lauryl-sarcosine	2 %
8-HESO; N-octyl-2-hydroxy-ethylsulfoxide	1.32 %
SDS; Sodium-N-dodecyl sulfate	0.1 %
<b>Zwitter-ionic Detergents</b>	
CHAPS	0.1 %
DDAO; N-decyl-N,N-dimethylamine-N-oxide	0.034 %
LDAO; N-dodecyl-N,N-dimethylamine-N-oxide	0.13 %
<b>Others</b>	
Ammonium sulfate (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2 M
CaCl <sub>2</sub>	max. 1 M
EDTA	50 mM
Ethanol	10 %
Guanidine	max. 1 M
Glycerol	max. 25 %
Imidazole	max. 250 mM
MgCl <sub>2</sub>	1 M
NaCl	5 M
Urea	max. 1 M

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

Note: These reagents have been successfully tested for the purification of e.g. GAPDH-*Strep*-tag with concentrations up to those mentioned. For reagents not marked with "max." higher concentrations may be possible, though. Since binding depends on the sterical accessibility of *Strep*-tag in the context of the particular protein the possible concentration may deviate from the given value for other proteins.

## 2 Cloning with pEXPR-IBA

### Cloning of an arbitrary gene into pEXPR-IBA expression vectors

The pEXPR-IBA vectors multiple cloning sites include many standard unique restriction sites like *EcoRI* or *BamHI* for the introduction of foreign genes after PCR. However, the reading frame of the corresponding vector has to be considered if such restriction sites are planned to be used. Using standard unique restriction sites, additional polylinker derived amino acids are appended at the respective end of the recombinant protein. To avoid the fusion of such polylinker derived amino acids pEXPR-IBA vectors offer a general cloning strategy via Type IIS restriction enzymes, *BsaI* or *Eco31I* (NEB, MBI Fermentas). They allow the precise fusion of the structural gene with the vector encoded functional elements (One-STrEP-tag and, depending on the vector, start codon (pEXPR-IBA103), or stop codon (pEXPR-IBA105)). To accomplish this it is necessary to adapt the structural gene at both ends of the coding region via PCR (cf. general cloning scheme in the IBA catalogue or [www.iba-go.com](http://www.iba-go.com)). The essential primer sequences to introduce the *BsaI* restriction site into the PCR fragment for the cloning with a defined pEXPR-IBA vector can be easily determined with our "Primer D'Signer Software" which is free of charge and can be downloaded at our web site. In order to avoid the incorporation of base substitutions, PCR should be performed with a proof reading DNA polymerase (e.g. *Pfu*, MBI Fermentas) using phosphorothioate (PTO) protected primers.

### PCR with *Pfu* DNA polymerase

Standard PCR assay; hot-start; PTO protected primers

Mix the following reagents in a 500  $\mu$ l reaction tube:

		Final concentration:	
dNTP (10 mM each)	1 $\mu$ l	200 $\mu$ M	
Forward primer (10 $\mu$ M)	2.5 $\mu$ l	500 nM	
Reverse primer (10 $\mu$ M)	2.5 $\mu$ l	500 nM	
10x buffer (supplier)	5 $\mu$ l		
Template DNA	X $\mu$ l	20 to 200 pg/ $\mu$ l	(plasmid DNA)
		0.1 to 1 ng/ $\mu$ l	(cDNA library)
H <sub>2</sub> O	ad 50 $\mu$ l		

Overlay the sample with 50  $\mu$ l mineral oil or use a cycler with heated lid and heat the sample at 94 °C for 3 min. Add 1  $\mu$ l *Pfu* DNA polymerase (2.5 u/ $\mu$ l) and start temperature cycling.

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

automatically if the “Primer D’Signer Software” is used). PCR annealing should be performed at 55 °C.

If plasmid DNA 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. 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, *Bsal* or *Eco311***

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 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<sub>2</sub>O, *Bsal* restriction can be performed immediately without any precipitation step.

The pEXPR-IBA vectors can be digested with the isoschizomers *Bsal* or *Eco311*. However, both enzymes show different cutting efficiencies in dependence of the DNA source (vector DNA or PCR fragment) and the incubation time. Therefore we performed a comparison of *Bsal* vs. *Eco311* and determined the cloning efficiency by counting the resulting colonies after transformation of the ligation reaction into *E. coli* DH5alpha cells. As a result, we recommend using *Bsal* for 1 hour or *Eco311* for 16 hours for the cleavage of both the PCR fragment and the vector.

			pASK-IBA3			
			<i>Bsal</i>		<i>Eco311</i>	
			1 h	16 h	1 h	16 h
PCR fragment	<i>Bsal</i>	1 h	<b>1208</b>	<b>1028</b>	<b>265</b>	<b>291</b>
		16 h	<b>92</b>	<b>51</b>	<b>22</b>	<b>10</b>
	<i>Eco311</i>	1 h	<b>77</b>	<b>2</b>	<b>12</b>	<b>8</b>
		16 h	<b>1271</b>	<b>1228</b>	<b>952</b>	<b>1140</b>
no PCR fragment (control)			<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>

**Table 2. Determination the cloning efficiency of a PCR fragment into pASK-IBA3 using *Bsal* or *EcoRI*.**

The vector pASK-IBA3 has been digested by *Bsal* and *Eco311* for 1 or 16 hours, respectively (see columns). To reduce background the linearized vector was dephosphorylated using shrimp alkaline phosphatase. The DNA has been purified via an agarose gel and was ligated to PCR fragments which have been digested in the same way (see rows). After overnight incubation at 16°C the ligation reaction was transformed into DH5alpha cells and plated onto LB/ampicillin plates. The resulting colonies were determined and are indicated in bold.

### Protocol

For restriction digest of the **PCR fragment** add 5 µl 10x *Bsal* (or *Eco311*) restriction buffer to the spin eluate, respectively.

Add H<sub>2</sub>O and restriction enzyme to 50 µl, using 10 to 20 units of the enzyme per µg DNA. Overlay with mineral oil or use a Thermo Cycler with heated lid and incubate at 50 °C with *Bsa*I for 1 h (or at 37°C with *Eco*31I for 16 h).

For restriction digest of the **vector** incubate 1-2 µg vector DNA with 10 to 20 units *Bsa*I at 50 °C for 1 hour (or *Eco*31I at 37 °C for 16 hours).

To reduce background after ligation which may result from re-ligated vector, dephosphorylate linearized vector DNA with phosphatase (e.g. shrimp alkaline phosphatase from USB) according to the manufacturer's 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<sub>2</sub>O ad 20 µl

Incubate overnight at 16 °C and store the sample at 4 °C until transformation.

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

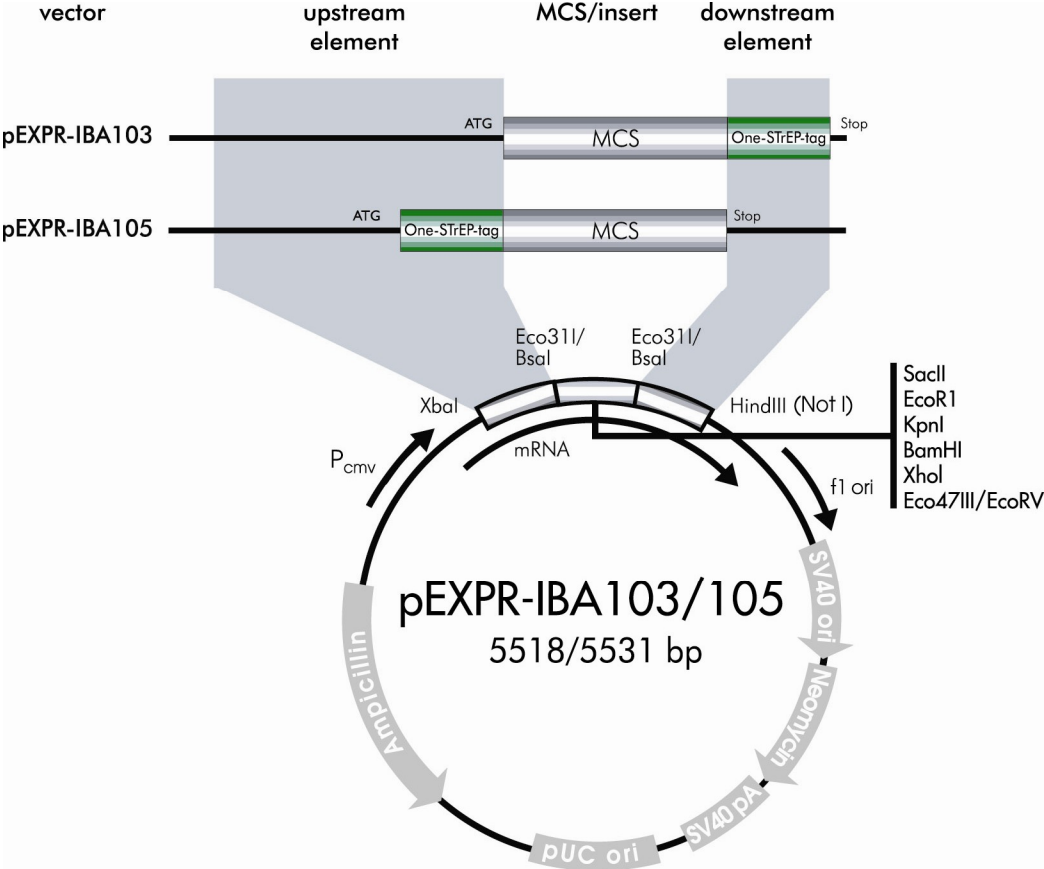
Sequencing primers for pEXPR-IBA vectors (order-no. 5-0000-123):

Forward: 5'-GAGAACCCACTGCTTACTGGC-3'

Reverse: 5'-TAGAAGGCACAGTCGAGG-3'

## 2.1 Description of pEXPR-IBA103 and pEXPR-IBA105

### 2.1.1 Map



## 2.1.2 Multiple cloning sites

### pEXPR-IBA103

CAAT  
GTCTCCACCCCATTTGACGTCAATGGGAGTTTGTGTTTGGCACCAAAAATCAACGGGACTTTCCAAAATGTCGTAACAACCTCCGCCCATTTGACG**CAAT**GGGGC

TATA forward primer  
GTAGGCGGTACGGTGGGAGGTT**TATA**TAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGG

**Eco31I PshAI** **PshAI Eco31I**  
XbaI **BsaI** SacII EcoRI KpnI BamHI XhoI **BsaI** Eco47III  
TCTAGACCCACaatgGGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATTGGTCTCagcgcTTGGAGC  
MetGlyAspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuSerAlaTrpSer  
Link

Kpn2I HindIII  
CACCCGAGTTCGAGAAAGGTGGAGGTTCCGGAGGTGGATCGGGAGGTGGATCGTGGAGCCACCCGAGTTCGAAAAATAATAAGCTTGCGGCCGAGATCT  
HisProGlnPheGluLysGlyGlySerGlyGlyGlySerTrpSerHisProGlnPheGluLysEnd  
One-StrEP-tag

reverse primer  
AGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGGCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCC

### pEXPR-IBA105

CAAT  
GTCTCCACCCCATTTGACGTCAATGGGAGTTTGTGTTTGGCACCAAAAATCAACGGGACTTTCCAAAATGTCGTAACAACCTCCGCCCATTTGACG**CAAT**GGGGC

TATA forward primer  
GTAGGCGGTACGGTGGGAGGTT**TATA**TAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGG

XbaI NheI Kpn2I  
TCTAGACCCACCATGGCTAGCTGGAGCCACCCGAGTTCGAGAAAGGTGGAGGTTCCGGAGGTGGATCGGGAGGTGGATCGTGGAGCCACCCGAGTTCGAA  
MetAlaSerTrpSerHisProGlnPheGluLysGlyGlySerGlyGlyGlySerTrpSerHisProGlnPheGlu  
Link One-StrEP-tag

**Eco31I PshAI** **PshAI Eco31I**  
**BsaI** SacII EcoRI KpnI BamHI XhoI **BsaI** EcoRV HindIII  
AAAGgcgcCGAGACCGCGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATTGGTCTCgataTCTAACTAAGCTTG  
LysGlyAlaGluThrAlaValProAsnSerSerSerValProGlyAspProSerArgSerThrCysArgGlyThrMetValSerAspIleEnd  
Link ArgProArgSerArgIleArgAlaArgTyrProGlyIleProArgGlyArgProAlaGlyGlyProTrpSerLeuIleSerAsnEnd  
AspArgGlyProGluPheGluLeuGlyThrArgGlySerLeuGluValAspLeuGlnGlyAspHisGlyLeuEnd

reverse primer  
CGGCCGAGATCTAGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGGCCCTCCCCCGTGCCTTCCTTGAC

#### Please note:

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

## 3 Expression

pEXPR-IBA vectors are designed for high-level stable as well as non-replicative transient expression in most mammalian cells by means of the constitutive CMV promoter. When using StarGate ([www.stargate-cloning.com](http://www.stargate-cloning.com)), further options are possible.

### 3.1 Transient expression in mammalian cells

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

- DNA purity
- host cell line used
- transfection method

Choose a method that will give the highest efficiency for the chosen cell line. Protocols for transfection can be found in [1]. An alternative method is presented below (3.1.1).

Depending on cell type and nature of the protein expression levels of 0.1 -1  $\mu\text{g}$  protein can be achieved per million cells (when low expression levels or transfection efficiency are expected, cell numbers should be increased accordingly), usually sufficient for detection with a specific antibody in total lysates (see chapter 5, page 19). Once expression is verified, transfection of approximately  $10^9$  cells in general allows expression and purification of protein from mammalian cells in the milligram range. In some cases, however, proteins cannot be expressed at high levels, especially when they are toxic for the cells. Please refer to section 3.3 on page 16 for hints. Cell lines latently infected with SV40 or expressing SV40 large T antigen like COS-1 and COS-7 cells allow episomal replication and thus longer expression periods.

#### Material and important notes

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

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

#### 3.1.1 Transfection with MATra

A large number of methods have been established for transient transfection. They differ in efficiency, cost and time requirements depending on the cell type used. Among these, magnet assisted transfection (MATra) is a new, easy-to-handle and highly efficient method to transfect cells in culture. Using this new technique nucleic acids, such as plasmid DNA, oligonucleotides or siRNA, are in a first step associated with magnetic particles. Exploiting magnetic force the full nucleic acid dose is then rapidly drawn towards and delivered into

the target cells leading to efficient transfection. Further Information on MATra is available at [www.magnet-assisted-transfection.com](http://www.magnet-assisted-transfection.com)

### 3.2 Stable expression in mammalian cells

pEXPR-IBA vectors contain a neomycin resistance gene to allow for selection of clones that have integrated the pEXPR-IBA DNA stably into their genome. Neomycin concentrations required need to be determined for each cell line. Determining expression levels in addition to Neomycin resistance is highly recommended. Depending on cell type and nature of the protein expression levels of 0.1 -1  $\mu$ g protein can be achieved per million cells, usually sufficient for detection with a specific antibody in total lysates (see chapter 5, page 19).

### 3.3 Trouble shooting – Expression

<b>Problem</b>	<b>Possible Cause</b>	<b>Comments and Suggestions</b>
Low expression levels or no expression	Sequence error, mutation	Verify sequence and reading frame
	Protein is toxic	Some proteins are inhibiting cell growth or induce apoptosis. In some cases signaling-inactive but binding competent forms can be expressed at high levels.
	Protein lacks co-factors	Try different cell types, when available cell types related to those expressing the bait protein in vivo (although: endogenous protein may compete with bait for binding partner, see chapter 4.1 page 18) known co-factors can be added to lysis buffer (e.g. metal ions)
Protein is secreted	Signal sequence present	Remove all signal sequences from the coding region.

## 4 Preparation of soluble cell extracts

### Material and important notes

- Only comparison of the preparation of untransfected (mock) versus transfected cells reveals the proteins bound specifically to the bait protein. Thus, two lysate preparations, one from cells after expression of the bait and one from untransfected control cells, are absolutely required.
- In general, addition of protease inhibitors is recommended. These are available against specific enzymes like trypsin or pepsin inhibitors or in cocktails. Add inhibitors immediately before use. Prepare only amount required for one day.
- If activity of enzymes needs to be preserved specific inhibitors may be required like phosphatase inhibitors for kinases and other signaling molecules.
- Reducing agents may be added to prevent non-specific disulfide cross-linking.
- Lysis buffers need to be tested for any expressed protein. One-STrEP-tag:*Strep*-Tactin binding is compatible with many reagents and detergents (see Table 1 on page 9).

### Buffers:

- Lysis Buffer (Buffer L): 50 mM Tris/HCl pH 7.4, 7.5 % glycerol, 150 mM NaCl, 1 mM EDTA
- It is recommended to work without EDTA when metalloproteins have been expressed or when the complex is suspected to be dependent on divalent ions. If Buffer L is intended to be used instead of preparing a new buffer, add CaCl<sub>2</sub> or MgCl<sub>2</sub> to Buffer L to a final concentration of 2 mM.
- 5x SDS-PAGE sample buffer: 0.25 M Tris/HCl, pH 8.0; 25% glycerol; 7.5% SDS, 0.25 mg/ml bromophenol blue; 12.5% v/v mercaptoethanol

### For the preparation of the control protein also required:

- Protease Inhibitors (Roche), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mM DTT; all added fresh to lysis buffer

#### 1. Chill Buffer L at 4°C.

#### 2. Thaw the cell pellet for 15 minutes on ice and resuspend the cells in Buffer L at 4 ml per 10<sup>9</sup> cells.

In case of metalloproteins or divalent ion dependent protein complexes, add CaCl<sub>2</sub> or MgCl<sub>2</sub> to Buffer L to a final concentration of 2 mM prior resuspension.

#### 3. Homogenize by 10 strokes with a tight-fitted Dounce homogenizer (keep on ice during the entire procedure).

Homogenizer can be washed with water and soap and sprayed with ethanol. Before use, rinse with distilled water.

#### 4. Centrifuge lysate at 750 x g for 10 minutes at 4°C to pellet the cellular debris.

5. **Add 5  $\mu$ l 5x SDS-PAGE sample buffer to 20  $\mu$ l supernatant and store at -20°C for SDS-PAGE analysis.**
6. **Centrifuge supernatant 1 hour at 100.000 x g and 4°C to prepare a lysate which contains the protein complex.**  
 Insoluble protein may clog the purification column in the following steps and needs to be removed.
7. **Proceed to protocols for One-STrEP-tagged protein purification under native conditions (see protocols 6 page 22).**

#### 4.1 Trouble shooting – Soluble Extracts

<b>Problem</b>	<b>Possible Cause</b>	<b>Comments and suggestions</b>
Bait protein is found in pellet	Protein is not soluble.	Stronger solubilizing lysis buffer and/or lysis conditions are required.
Protein is degraded	Cell lysis is too harsh releasing endosomal proteases, or protein is very susceptible to protease digestion.	Addition of protease inhibitors is generally recommended. Milder solubilizing conditions should be tested. Where possible, removal of destabilizing sequences greatly increases half-life.

## 5 Protein Detection

### 5.1 Chemoluminescence detection of *Strep*-tag proteins with *Strep*MAB-Classic horse radish peroxidase (HRP) conjugate

#### Material and important notes

- PBS buffer: 4 mM  $\text{KH}_2\text{PO}_4$ , 16 mM  $\text{Na}_2\text{HPO}_4$ , 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)
- Chemoluminescence detection solution (not included)

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 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. **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  $\mu\text{l}$  (after blocking with 1% milk powder in PBS-Tween add 2.5  $\mu\text{l}$  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).**

#### Chemiluminescent Detection

**Follow the manufacturer's protocol provided with the respective reagents**

## 5.2 Detection of PP2Ac with specific polyclonal control antibody

To assist in trouble shooting, all reagents required to carry out and detect a protein-protein interaction were included. A control vector containing the coding sequence of PR65 fused to One-STrEP-tag allows expression of this bait in the cell line of choice (human cell lines like HT1080, 293 or HeLa are recommended). PR65 is a structural subunit of the PP2A phosphatase holoenzyme and thus should bind PP2Ac, the respective catalytic subunit leading to its co-purification. Using the polyclonal antibodies directed against PP2Ac in the kit, this protein complex can be verified by Western blotting.

### Material and important notes

- Use affinity-purified goat polyclonal antibody solution diluted in PBSMT blocking buffer 1:1000
- PBS buffer: 4 mM  $\text{KH}_2\text{PO}_4$ , 16 mM  $\text{Na}_2\text{HPO}_4$ , 115 mM NaCl, pH 7.4
- PBSMT blocking buffer: PBS buffer with 5 % nonfat dry milk and 0.1 % v/v Tween 20
- PBS-Tween buffer: PBS buffer with 0.1 % v/v Tween 20
- Control antibody: Goat anti PP2Ac polyclonal antibody, working dilution 1:1000 in PBSMT blocking buffer
- Rabbit anti-goat antibodies HRP conjugated (e.g. DAKO cat. no. P0449), working dilution 1:2000 in PBSMT blocking buffer
- Chemiluminescence reagents and X-Ray films or:
- Chloronaphtol solution: 3 % w/v 4-chloro-1-naphtol in methanol
- $\text{H}_2\text{O}_2$  solution: 30 % v/v  $\text{H}_2\text{O}_2$
- **Note:** chromogenic reaction may not be sensitive enough to detect bound protein

1. **After SDS-PAGE and electrotransfer of the protein to an appropriate membrane block the membrane with 20 ml PBSMT-blocking buffer. Incubate 1 h (room temperature; with gentle shaking) or overnight (4°C).**  
We recommend using a nitrocellulose membrane.
2. **Wash 3 times with 20 ml PBS-Tween buffer (1x 10 minutes and 2x 5 minutes, room temperature, gentle shaking).**
3. **Discard washing buffer and add PP2Ac-specific goat polyclonal antibody diluted 1:1000 in PBSMT blocking buffer.**
4. **Incubate 60 minutes at room temperature, gentle shaking.**
5. **Wash 3 times with 20 ml PBS-Tween buffer (1x 10 minutes and 2x 5 minutes, room temperature, gentle shaking).**
6. **Discard washing buffer and add HRP conjugated rabbit anti goat polyclonal antibody diluted 1:2000 in PBSMT blocking buffer.**
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) and proceed to detection.

#### **Chemiluminescent Detection**

Follow the manufacturer's protocol provided with the respective reagents.

#### **Or (optional) Chromogenic Detection**

9. Transfer membrane in 20 ml PBS buffer, add 200  $\mu$ l chloronaphtol solution and 20  $\mu$ l  $H_2O_2$  solution.

NOTE: Sensitivity of chromogenic detection may not be sufficient to detect PP2Ac.

10. Proceed 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.

## 6 Purification of One-STrEP-tag fusion proteins

### 6.1 Purification of One-STrEP-tag fusion proteins using gravity flow columns

#### Material and important notes

- **CV = column bed volume**
- *Strep-Tactin Superflow* can be used for gravity flow purification
- Binding capacity of each matrix is 50 - 100 nmol recombinant protein per ml bed volume (100 nmol correspond to 3 mg of a 30 kDa protein)
- Buffer W (washing buffer): 100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 8
- Buffer BE (biotin elution buffer): 100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 2 mM biotin, pH 8
- The composition of the lysis, wash and elution buffers may have to be modified to solubilize the bait protein while preserving the respective interactions, e.g. by adding 0.1% Tween, 5-10 mM  $\beta$ -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 9. When a metalloprotein is used as bait or when the protein complex to be isolated is dependent on divalent ions, add  $\text{CaCl}_2$  or  $\text{MgCl}_2$  to Buffer W and Buffer BE to an end concentration of 2 mM (use a stock solution having a concentration of at least 200 mM).
- 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	1 - 4 ml	5 x 0.2 ml	6 x 0.1 ml
1 ml	4 - 12 ml	5 x 1 ml	6 x 0.5 ml

**Table 3: Recommended buffer volumes for chromatography on *Strep-Tactin* columns**

\*Adjust protein extract volume according to binding capacity of the column and apply the extract as concentrated as possible in the recommended volume range.

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

Storage buffer is removed prior to equilibration.

The column cannot run dry under gravity flow.

In case of metalloproteins or divalent ion dependent protein complexes use buffer without EDTA or add  $\text{CaCl}_2$  or  $\text{MgCl}_2$  to Buffer W to an end concentration of 2 mM (use a stock solution having a concentration of at least 200 mM).

## **2. Add soluble cell extracts to the column.**

The volume of the cell extracts should be in the range of 1 to 20 CVs (see Table on page 22). Extracts of large volumes with the recombinant protein at low concentration may lead to reduced yields and should be concentrated prior to chromatography.

Concentrated cell extracts are preferred; if quantification is possible, apply cell extract containing between 50 and 100 nmol recombinant One-STrEP-tag fusion protein per 1 ml CV.

## **3. Wash the column 5 times with 1 CV Buffer W, after the cell extract has completely entered the column.**

Collect the flow-through in fractions of 1 CV. Apply 2  $\mu$ l of the first washing fraction and 20  $\mu$ l of each subsequent fraction to an analytical SDS-PAGE. In case of metalloproteins or divalent ion dependent protein complexes use buffer without EDTA or add  $\text{CaCl}_2$  or  $\text{MgCl}_2$  to Buffer W to an end concentration of 2 mM (use a stock solution having a concentration of at least 200 mM).

## **4. Add 6 times 0.5 CVs Buffer BE and collect the eluate in 0.5 CV fractions.**

20  $\mu$ l samples of each fraction can be used for SDS-PAGE analysis. The purified One-STrEP-tag fusion protein usually elutes in the 2<sup>nd</sup> to 5<sup>th</sup> fraction. In case of metalloproteins or divalent ion dependent protein complexes use buffer without EDTA or add  $\text{CaCl}_2$  or  $\text{MgCl}_2$  to Buffer BE to an end concentration of 2 mM (use a stock solution having a concentration of at least 200 mM).

### **For protein identification on silver gels**

- 5. Identify the fractions containing the bait protein by SDS-PAGE/western blot using *StrepMAB*-Classic (see chapter 5.1).**
- 6. Pool the positive fractions, concentrate them using ultra-filtration units (i.e. Amicon, VivaScience) to maximize loading and run them on a denaturing polyacrylamide gel.**
- 7. Excise bands not present in mock (untransfected) control lysates for analysis.**

## 6.2 Trouble shooting – One-STrEP-tag purification

### 6.2.1 “No or weak binding to *Strep*-Tactin column”

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

### 6.2.2 “Too many background proteins are bound and eluted”

Non-specific proteins	Add additives to the buffers. Refer to Table 1 where many reagents are listed that may reduce non specific binding to the resin or bait and which are compatible with One-STrEP-tag binding. Ionic interactions may be reduced by increasing the ionic strength; hydrophobic interactions may be reduced by adding detergents and disulfide bridge induced covalent interactions may be reduced by adding reducing reagents. Conditions should not be such that specific complexes are broken up.
Biotinylated host proteins	May be verified by Western blotting and immunodetection with <i>Strep</i> -Tactin HRP or AP conjugate (Cat. No. 2-1502-001 or 2-1503-001 respectively). Add a slight excess (with respect to biotinylated contaminations) of avidin (Cat. No. 2-0204-015) to the cell extract. Incubate the extract for at least 15 minutes with avidin and centrifuge again (microfuge, max speed, 5 minutes) immediately prior to subjecting the extract to affinity chromatography to remove precipitated biotinylated proteins.

### 6.2.3 “No/not enough interacting proteins found”

Co-factors required	If bait protein is binding efficiently under mild conditions interacting proteins should not be washed off unless they require specific co-factors or modifications. If the protein complex is known or suspected to be cation binding or phosphorylated for example, try adding $\text{Ca}^{2+}$ and/or $\text{Mg}^{2+}$ (> 1 mM; excess towards EDTA (1 mM) present in all buffers is needed) the or add phosphatase inhibitors, respectively.
Competition with endogenous protein	When endogenous untagged protein is present, it competes with tagged bait for binding partners. Specific knock-down with siRNA directed against endogenous protein (using untranslated sequences not used in transfection plasmid) will increase binding efficiency.

### 6.2.4 “Bubbles in the column”

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

Bubbles developing in the column bed.	Keep on working in the cold room (recommended for protein purification), use degassed buffers or wash the column immediately with buffers equilibrated at ambient temperature once the column is removed from the cold.
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## 7 APPENDIX

### 7.1 Storage of *Strep*-Tactin resin

#### Material and important notes

- *Strep*-Tactin matrices should be stored at 2 – 8 °C.
- Buffer W: 100 mM Tris/HCl, 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.

### 7.2 Ordering information

Cat. no.	Product
2-1109-000	One-STrEP Starter Kit
2-1110-000	One-STrEP Follow-up Kit (without controls)

### 7.3 Related products

Cat. no.	Product
2-0204-015	Avidin; 15 mg
2-0501-002	Biotin Blocking Buffer; 2 ml
2-1011-100	<i>Strep</i> -tag® Protein Ladder
2-1206-010	<i>Strep</i> -Tactin® Superflow®; 20 ml 50 % suspension
2-1206-025	<i>Strep</i> -Tactin® Superflow®; 50 ml 50 % suspension
2-1206-100	<i>Strep</i> -Tactin® Superflow®; 200 ml 50 % suspension
2-1206-500	<i>Strep</i> -Tactin® Superflow®; 1000 ml 50 % suspension
2-1207-001	Gravity flow <i>Strep</i> -Tactin® Superflow® column; 1 x 1 ml
2-1207-005	Gravity flow <i>Strep</i> -Tactin® Superflow® column; 5 x 1 ml
2-1207-505	Gravity flow <i>Strep</i> -Tactin® Superflow® column; 5 x 0.2 ml
2-1502-001	<i>Strep</i> -Tactin® HRP conjugate; 0.5 ml
2-1503-001	<i>Strep</i> -Tactin® AP conjugate; 0.5 ml
2-1509-001	<i>Strep</i> MAB-Classic HRP conjugate

Cat. no.	Product
2-1526-001	Gravity flow <i>Strep</i> MAB-Classic MacroPrep® column 1 x 1 ml
2-1526-505	Gravity flow <i>Strep</i> MAB-Classic MacroPrep® column 5 x 0,2 ml
5-0000-121	Forward sequencing primer for pEXPR-IBA; 1 nmol
5-0000-122	Reverse sequencing primer for pEXPR-IBA; 1 nmol
5-0000-123	Forw. and rev. seq. primers for pEXPR-IBA; 1 nmol each
7-2001-020	MATra-A Reagent for 200 µg nucleic acids
7-2001-100	MATra-A Reagent for 1000 µg nucleic acids
7-2002-020	MATra-S Immobilizer up to 7 Mio. cells
7-2002-100	MATra-S Immobilizer up to 35 Mio. cells
7-2003-020	MA Lipofection Enhancer up to 200 µg nucleic acids
7-2003-100	MA Lipofection Enhancer up to 1000 µg nucleic acids
7-2004-000	96 Magnet Plate

## 8 References

**For up-to-date references see [www.iba-go.com](http://www.iba-go.com)**

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