One-STrEP™ Kit

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

A comprehensive manual

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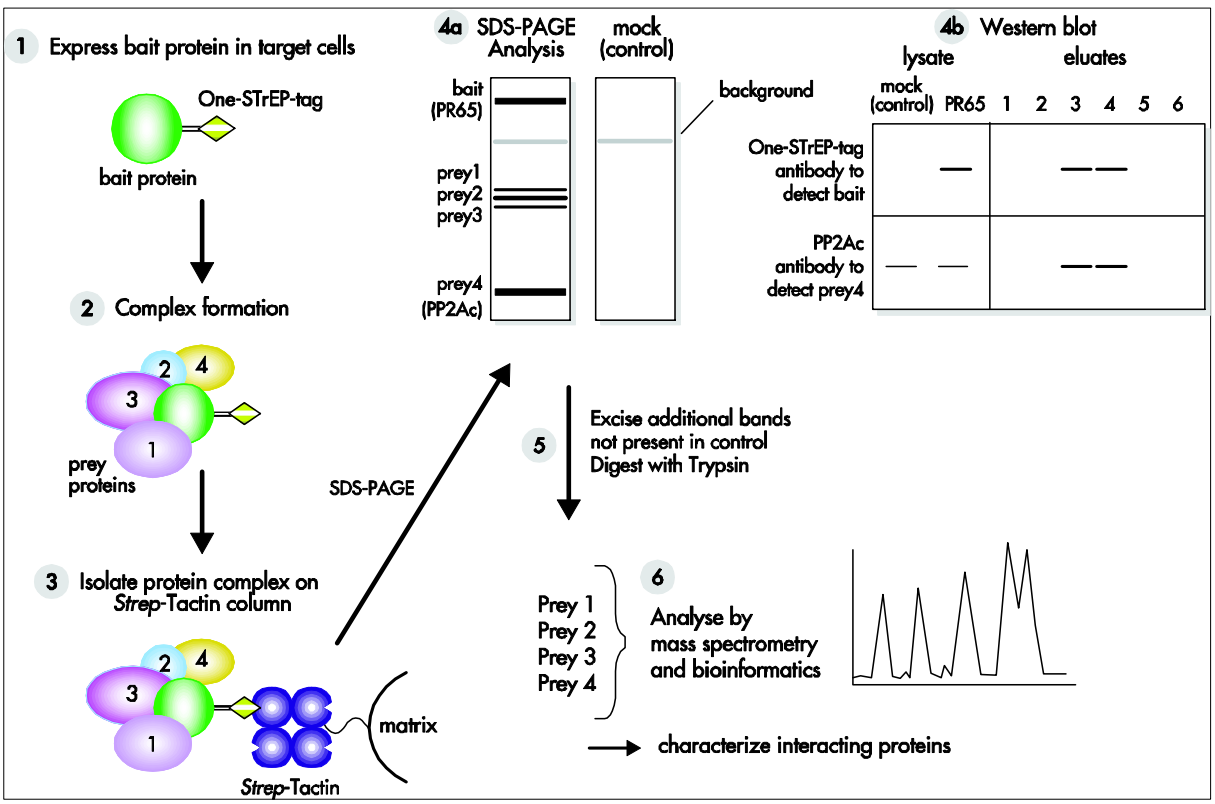
Content

1 Introduction

1.1 Protein-Protein Interactions

After the sequencing of the human genome the identification of protein functions remains the great challenge in biological sciences. Here, protein-protein interactions are a key element in elucidating the intricate cellular networks. While genetic methods like yeast two-hybrid screens [1] allow large scale systematic analysis, they also yield a substantial amount of false results. In addition, interactions requiring more than two partners or co-factors e.g. the authentic cellular environment of the molecules are not found in yeast. To overcome these limitations, pull-down assays in mammalian cells have been used. However, they require a tedious optimization of binding and washing conditions because of the high purification background they produce. An improvement to this method was the tandem affinity purification (TAP) [2].
While TAP requires two successive purification steps, the One-STrEP kit makes use of the extremely fast and efficient One-STrEP-tag/*Strep*-Tactin system. This system yields minimum background after just one purification step [3,4]. Furthermore, an one-step procedure reduces sample dilution which together with the short time required for the entire process, prevents the loss of transient and weak interactions [5]. Being a peptide tag the One-STrEP-tag is presumed to minimally interfere with complex formation.



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-STrEP-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 form the gel and subsequent mass spectrometric analysis (5, 6) or, if interaction partners are known or expected, by Western blot using specific antibodies (4b).

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, a One-STrEP-tag monoclonal antibody conjugated to horse radish peroxidase is included.
As a control for the entire procedure, a control plasmid encoding a bait and antibodies for 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 monoclonal antibody 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 [3].

1.2 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.

1.3 One-STrEP-tag/*Strep*-Tactin® 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 [6,7,8].
After application of the crude extract to a *Strep*-Tactin column and a short washing step, gentle elution of recombinant bait and all bound proteins is performed by addition of low concentrations of biotin (2 mM). The One-STrEP-tag/*Strep*-Tactin interaction is compatible with a variety of reagents (see Table 1).
Comprehensive reviews and scientific publications giving an overview of various *Strep*-tag applications are listed at www.iba-go.com.

Reagent	Concentration
Reducing Agents	
DTT	50 mM
β-mercaptoethanol	50 mM
Non-Ionic Detergents	
C_8E_4 Octyltetraoxyethylene	0.88 %
$C_{10}E_5$; Decylpentaoxyethylene	0.12 %
$C_{10}E_6$	0.03 %
$C_{12}E_8$	0.005 %
$C_{12}E_9$; Dodecylnonaoxyethylene (Thesit)	0.023 %
DM; Decyl-ß-D-maltoside	0.35 %
LM; N-dodecyl-β-D-maltoside	0.007 %
NG; N-nonyl-β-D-glucopyranoside	0.2 %
OG; N-octyl-β-D-glucopyranoside	2.34 %
TX; Triton X-100	2 %
Tween 20	2 %
NP-40, nonidet P40/Octylphenolpoly(ethylenglycolether)	0.5 %
Ionic Detergents	
N-lauryl-sarcosine	2 %
8-HESO;N-octyl-2-hydroxy-ethylsulfoxide	1,32 %
SDS; Sodium-N-dodecylsulfate	0.1 %
Zwitter-Ionic Detergents	
CHAPS	0.1 %
DDAO; N-decyl-N,N-dimethylamine-N-oxide	0.034 %
LDAO; N-dodecyl-N,N-dimethylamine-N-oxide	0.13 %
Others	
Ammonium sulfate $(NH_4)_2SO_4$	2 M
$CaCl_2$	1 M
EDTA	50 mM
Ethanol	10 %
Guanidine	1 M
Glycerol	25 %
Imidazole	250 mM
$MgCl_2$	1 M
NaCl	5 M
Urea	1 M

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

Note: These reagents have been successfully tested for the purification of e.g. GAPDH-*Strep*-tag with concentrations up to those mentioned. For most reagents higher concentrations may be possible, though. However, since binding depends on the sterical accessibility of *Strep*-tag in the context of the particular protein the maximal concentration may vary 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 *Eco*RI or *Bam*HI for the introduction of foreign genes after PCR. However, the reading frame of the corresponding vector has to be considered if such restriction sites are planned to be used. Using standard unique restriction sites, additional polylinker derived amino acids are appended at the respective end of the recombinant protein. To avoid the fusion of such polylinker derived amino acids pEXPR-IBA vectors offer a general cloning strategy via Type IIS restriction enzymes, *Bsa*I or *Eco*31I (NEB, MBI Fermentas). They allow the precise fusion of the structural gene with the vector encoded functional elements (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 (s. general cloning scheme in the IBA catalogue or at www.iba-go.com). The essential primer sequences to introduce the *Bsa*I restriction site into the PCR fragment for the cloning with a defined pEXPR-IBA vector can be easily determined with our "Primer D'Signer Software" which is free of charge and can be downloaded at our web site. In order to avoid the incorporation of base substitutions, PCR should be performed with a proof reading DNA polymerase (e.g. *Pfu*, MBI Fermentas) using phosphorothioate (PTO) protected primers.

PCR with *Pfu* DNA polymerase

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

		Final concentration:	
dNTP (10 mM each)	1 µl	200 µM	
Forward primer (10 µM)	2.5 µl	500 nM	
Reverse primer (10 µM)	2.5 µl	500 nM	
10x buffer (supplier)	5 µl		
Template DNA	X µl	20 to 200 pg/µl	(plasmid DNA)
		0.1 to 1 ng/µl	(cDNA library)
H_2O	ad 50 µl		

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

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

If plasmid DNA 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, *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 without prior separation on an agarose gel. Otherwise, a preparative agarose gel is essential for purification. If a spin kit is used and the DNA fragment is eluted in H_2O, *Bsa*I restriction can be performed immediately without any precipitation step.

The pEXPR-IBA vectors can be digested with the isoschizomers *Bsa*I or *Eco*31I. However, both enzymes show different cutting efficiencies in dependence of the DNA source (vector DNA or PCR fragment) and the incubation time. Therefore we performed a comparison of *Bsa*I vs. *Eco*31I and determined the cloning efficiency by counting the resulting colonies after transformation of the ligation reaction into *E. coli* DH5alpha cells. As a result, we recommend using *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 2. Determination the cloning efficiency of a PCR fragment into pASK-IBA3 using *Bsa*I or *Eco*RI.
The vector pASK-IBA3 has been digested by *Bsa*I and *Eco*31I for 1 or 16 hours, respectively (see columns). To reduce background the linerized vector was dephosphorylated using shrimp alkaline phosphatase. The DNA has been purified via an agarose gel and was ligated to PCR fragments which have been digested in the same way (see rows). After overnight incubation at 16°C the ligation reaction was transformed into DH5alpha cells and plated onto LB/ampicillin plates. The resulting colonies were determined and are indicated in bold.

Protocol

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

For restriction digest of the **vector** incubate 1-2 µg vector DNA with 10 to 20 units *Bsa*I at 50 °C for 1 hour (or *Eco*31I at 37 °C for 16 hours).
To reduce background after ligation which may result from re-ligated vector, dephosphorylate linerized vector DNA with phosphatase (e.g. shrimp alkaline phosphatase from USB) according to the 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_2O ad 20 µl
Incubate overnight at 16 °C and store the sample at 4 °C until transformation.

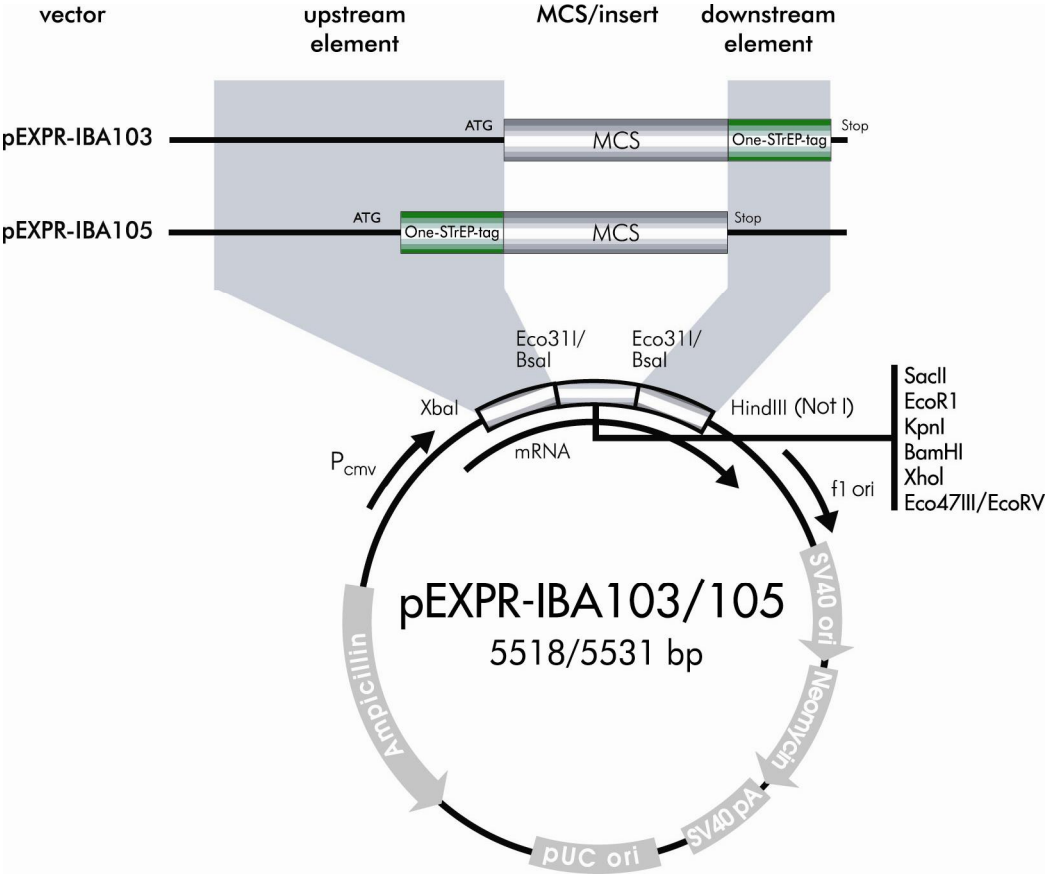
Simultaneously, perform the same ligation assay without the addition of PCR fragment for quantifying background reactions. After transformation and screening for a putative correct clone by DNA mini preparation (Biometra order no. 4100-450B) and subsequent restriction analysis, proceed to DNA sequencing.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

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

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

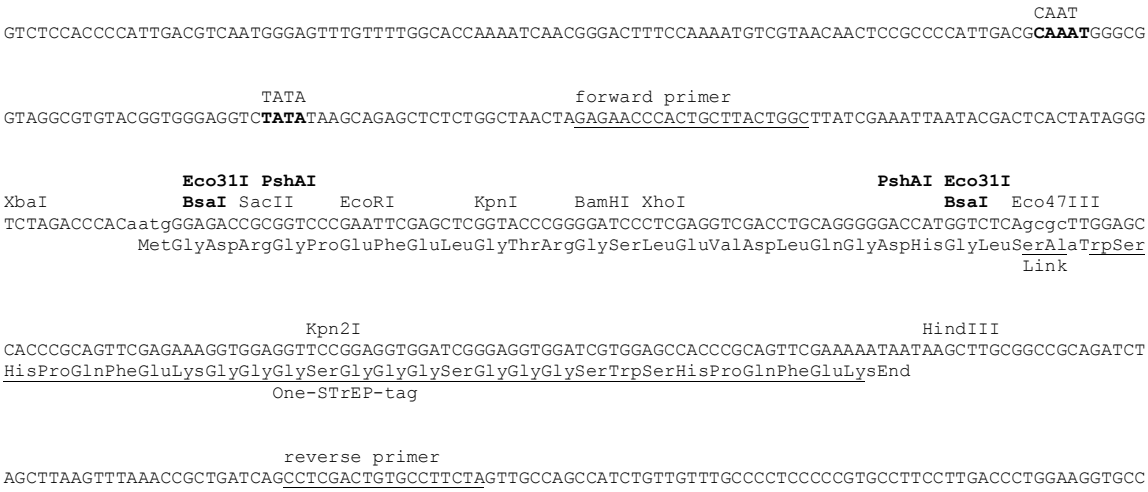
2.1 Description of pEXPR-IBA103 and pEXPR-IBA105

2.1.1 Map

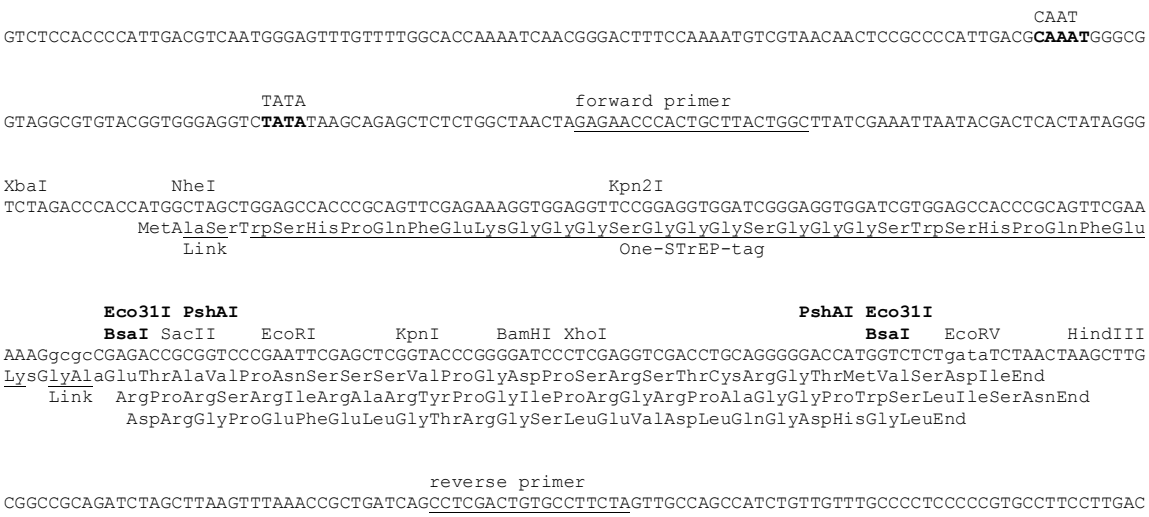


2.1.2 Multiple cloning sites

pEXPR-IBA103



pEXPR-IBA105



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.

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 [9]. An alternative method is presented below (3.1.1 page 12).

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

Material and important notes

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

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

3.1.1 Transfection 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 [9]. 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

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

magnetic force the full nucleic acid dose is then rapidly drawn towards and delivered into the target cells leading to efficient transfection. Further Information on MATra is available at www.magnet-assisted-transfection.com

3.2 Stable expression in mammalian cells

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

3.3 Trouble shooting – Expression

Problem	Possible Cause	Comments and Suggestions
Low expression levels or no expression	Sequence error, mutation	Verify sequence and reading frame
	Protein is toxic	Some proteins are inhibiting cell growth or induce apoptosis. In some cases signaling-inactive 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 15) 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 6).

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 $CaCl_2$ or $MgCl_2$ 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_3VO_4, 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 10^9 cells.**

   In case of metalloproteins or divalent ion dependent protein complexes, add $CaCl_2$ or $MgCl_2$ 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 µl 5x SDS-PAGE sample buffer to 20 µl 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 19).**

4.1 Trouble shooting – Soluble Extracts

Problem	Possible Cause	Comments and suggestions
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 Detection of *Strep*-tag proteins with *Strep*-tag II specific monoclonal antibody conjugated to 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 electrotransfer of the protein to an appropriate membrane block the membrane with 20 ml PBS-blocking buffer. Incubate: 1 h (room temperature; with gentle shaking) or overnight (4°C).**

   Do not use milk powder for blocking, because milk is one of the richest sources of biotin!
   We recommend 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. **Add 2.5 µl *Strep*-tag II specific monoclonal antibody horse radish peroxidase conjugate (1:4000). 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) and proceed to detection.**

Chemiluminescent Detection
Follow the manufacturer's protocol provided with the respective reagents

Chromogenic Detection
Please note that chromogenic detection is not as sensitive as chemiluminescence detection. Therefore, for some studies chemiluminescence detection might be a prerequisite.

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

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

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

10. **Air dry the membrane and store in the dark.**

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 KH_2PO_4, 16 mM Na_2HPO_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
- Chemiluminiscence reagents and X-Ray films or:
- Chloronaphtol solution: 3 % w/v 4-chloro-1-naphtol in methanol
- H_2O_2 solution: 30 % v/v H_2O_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 µl chloronaphtol solution and 20 µl H_20_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 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. When a metalloprotein is used as bait or when the protein complex to be isolated is dependent on divalent ions, add $CaCl_2$ or $MgCl_2$ 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 $CaCl_2$ or $MgCl_2$ 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 19). 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 µl of the first washing fraction and 20 µl 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 $CaCl_2$ or $MgCl_2$ 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 µl samples of each fraction can be used for SDS-PAGE analysis. The purified One-STrEP-tag fusion protein usually elutes in the 2nd to 5th fraction. In case of metalloproteins or divalent ion dependent protein complexes use buffer without EDTA or add $CaCl_2$ or $MgCl_2$ 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 the *Strep*-Tag antibody (see chapter 5.1 page 16).

6. Pool the positive fractions, concentrate them using ultra-filtration units (i.e. Amicon, VivaScience) to maximize loading and run them on a denaturing polycrylamide 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 *Strep*-tag with the other protein terminus; use other linker.
One-STrEP-tag has been degraded.	Check that the *Strep*-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.
Strep-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 Strep-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 Ca^{2+} and/or Mg^{2+} (> 1 mM; excess towards EDTA (1 mM) present in all buffers ist 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.

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
2-0501-002	Biotin Blocking Buffer; 2 ml
2-1011-100	*Strep*-tag® Protein Ladder
2-1206-010	*Strep*-Tactin® Superflow®; 20 ml 50 % suspension
2-1206-025	*Strep*-Tactin® Superflow®; 50 ml 50 % suspension
2-1206-100	*Strep*-Tactin® Superflow®; 200 ml 50 % suspension
2-1206-500	*Strep*-Tactin® Superflow®; 1000 ml 50 % suspension
2-1207-001	Gravity flow *Strep*-Tactin® Superflow® column; 1 x 1 ml
2-1207-005	Gravity flow *Strep*-Tactin® Superflow® column; 5 x 1 ml
2-1207-505	Gravity flow *Strep*-Tactin® Superflow® column; 5 x 0.2 ml
2-1509-001	*Strep*-tag II specific monoclonal antibody, HRP conjugate

Cat. no.	Product
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

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