Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 786 (2003) 343–355
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Semi automated production of a set of different recombinant GST-Streptag fusion proteins

Petra Sebastian, Jelena Wallwitz, Stefan Schmidt*

Department of Protein Biochemistry, GPC-Biotech AG, Fraunhoferstr. 20, D-82152 Martinsried, Germany

Abstract

We describe a high-throughput procedure for the large-scale production of recombinant GST-Streptag fusion proteins. This three-step process, comprising cloning, expression and purification, simultaneously produces up to 96 different proteins in a multi-well format with high yield and purity. Two complementary oligonucleotides, together encoding a specific peptide sequence are annealed and directly ligated into a pre-digested pGEX-2T plasmid carrying an N-terminal GST-tag and a C-terminal Streptag. Following expression, a multichannel pipetting robot purifies the resulting fusion proteins within 2 h by affinity chromatography on Streptactin Macroprep mini-columns.
© 2002 Published by Elsevier Science B.V.

Keywords: Automated purification; Streptag; Fusion proteins

1. Introduction

In the post genomic era the number of complete genome sequences, ranging from bacteria like *E. coli*, *B. subtilis*, pathogenic strains to *S. cerevisiae*, model organisms like *C. elegans* and *D. melanogaster* up to *H. sapiens*, already reached more than 800 until March 2002 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome). Building on this, one central goal in drug discovery lies in the functional characterization of their encoded gene products: the proteins [1].

The prerequisites for this approach comprise not only the efficient cloning of open reading frames or putative genes, which is now easily feasible by using a directed PCR cloning approach, but also an efficient expression vector with a selective affinity tag and finally a large-scale expression and purification procedure. Until now efforts have been undertaken to express the complete genome of an archaeon [2] and bakers yeast [3]. Recently the parallel expression of IMAGE clones in a baculovirus system was described [4,5]. The primary screen to test the solubility of expressed clones on a larger scale in the well-established 96-well format was presented by Knaust and Nordlund in October 2001 [6].

The challenges of high-throughput purification, primarily the low expression rates resulting from insolubility, toxicity to host organisms or unusual codon usage have been summarized lately in a number of reviews [7–10]. Once large numbers of novel purified proteins are available, a multitude of analytical possibilities emerge. These proteins can be used for structural crystallisation studies, for the generation of antibodies and for a completely new technological field, the analysis of proteins on microarrays [11]. Techniques have to be established allowing the simultaneous functional analysis of

*Corresponding author. Tel.: +46-8-552-53188; fax: +46-8-552-53189.
E-mail address: stefan.schmidt@astrazeneca.com (S. Schmidt).

1570-0232/02/$ – see front matter © 2002 Published by Elsevier Science B.V.
doi:10.1016/S1570-0232(02)00915-7

hundreds or even thousands of proteins in parallel. Ideally the analysis is performed in a format that minimizes sample consumption and is compatible with different assay conditions. Interesting assays are those interrogating any kind of interaction with other molecules like drugs, other proteins, antibodies and DNA, or those deducing biochemical functions of proteins. The latter are particularly important to investigate activities of pharmacologically relevant enzyme classes like kinases, phosphatases and proteases or enzymes used in biotechnological processes. Suitable analysis systems for this kind of application are, for instance, microarrays consisting of a solid support and immobilized purified proteins. The first example of such a global approach was a biochemical screen of all yeast ORFs [12].

We have focused on the generation of a set of kinase substrate fusion peptides, which are later immobilized on protein arrays to profile the activity of different kinases. The use of short peptide sequences required the fusion to a larger scaffold protein, thus preventing the rapid degradation of peptide fragments immediately after expression in the host. For that purpose a standard glutathione *S*-transferase (GST)-containing vector [13], pGEX-2T, was employed as the expression vector.

The most efficient purification method for recombinant proteins is the affinity chromatography. Since sufficient purity of the produced protein can be reached in a single chromatographic step, affinity purification is suitable for automation and high-throughput processing. Current purification approaches comprise liquid chromatographic systems as well as filtration devices, or other small-scale methods using magnetic beads. A critical parameter for all these approaches is the processing rate, which is rate-limiting for the achievable throughput. Single-channel systems like classical HPLC or FPLC devices are useful for the purification of a limited number of samples only. If a given approach, e.g., the generation of protein arrays, depends on a high sample throughput, multichannel systems like liquid handling robots should be acquired. The use of a multichannel pipetting robot in combination with filter plates containing the chromatographic matrix (a common system for the large scale preparation of plasmid DNA) is suited for high-throughput protein purification as well. For recombinant proteins carrying poly-histidine (His-tag) a similar approach is already commercially available (Qiagen, Hilden, Germany).

Out of a large number of potential affinity tags (e.g. His-tag, S-tag, Flag-tag, dihydrofolate reductase (DHFR) or maltose binding protein (MBP)), we have chosen the Streptag for its unique features: high selectivity, mild elution and the possibility to directly regenerate the chromatographic resin [14]. For that purpose the pGEX 2T expression vector was equipped with the Streptag on the C-terminal end of the expression cassette. We show that our semi-automated method allows the time- and cost-efficient high-throughput cloning, expression and purification of a large number of different fusion proteins.

2. Experimental

2.1. Cloning procedures

All recombinant DNA manipulations were performed according to standard procedures [15]. Restriction endonucleases and DNA ligase were used according to the manufacturer's instructions.

A modified pGEX-2T plasmid (Amersham–Pharmacia, Uppsala, Sweden) carrying an N-terminal GST-tag and a C-terminal Streptag was used as vector for introducing DNA fragments. In order to avoid an additional step requiring a restriction enzymatic (RE) digest, the oligonucleotides which encode the specific peptide sequences of kinase substrates were modified at the 3′ and 5′ end to result in four nucleotide protrusions which are compatible to the RE sites in the *Bam*HI–*Xho*I (New England Biolabs, Frankfurt, Germany) pre-digested vector. At the 5′ end of the coding oligonucleotide the sequence GATCC was fused and C at the 3′ end. The complementary oligonucleotide was modified by attaching TCGAG at the 5′ end and G at the 3′ end (see Table 1). The complementary oligonucleotides were annealed with 25 μ*M* of each in Tris–buffer (10 m*M* Tris–HCl, pH 7.5, 25 m*M* NaCl) in a total reaction volume of 20 μl. The DNA solutions were incubated at 96 °C for 10 min and cooled to room temperature within 3 h. The resultant DNA fragments were directly ligated into the *Bam*HI–*Xho*I pre-digested modified pGEX-2T plasmid. Chemical-

Table 1
Expression clones with sequences of insert DNA and encoded peptide sequence

No.	Names	AA sequence	DNA sequence (forward oligo 5′ + GATCC (= *Bam*HI), 3′ + C, reverse oligo 5′ + TCGAG (= *Xho*I); 3′ + G)
1	PKC31	RRRRRKGSFRRKA	CGCCGTCGCCGTCGCAAAGGCAGCTTTCGCCGTAAAGCG
2	ctrl	STOP	
3	pp60	RRLIEDAEYAARG	CGTCGTCTGATTGAAGATGCGGAATATGCGGCGCGTGGC
4	ACM	KKALRRQETVDAL	AAAAAAGCGCTGCGTCGTCAGGAAACCGTGGATGCGCTG
5	PKG	RKISASEFDRPLR	CGTAAAATTAGCGCGAGCGAATTTGATCGTCCGCTGCGT
6	IRO	TRDIYETDYYRL	ACCCGTGATATTTATGAAACCGATTATTATCGTCTG
7	TK2A	EEEEAYGWLDF	GAAGAAGAAGAAGCGTATGGCTGGCTGGATTTT
8	CDK1	HATPPKKKRK	CATGCGACCCCGCCGAAAAAAAAACGTAAA
9	CKII	RREEETEEE	CGTCGTGAAGAAGAAACCGAAGAAGAA
10	MBP	APRTPGGRR	GCGCCGCGTACCCCGGGCGGCCGTCGT
11	H1-7	RRKASGP	CGTCGTAAAGCGAGCGGCCCG
12	PKCsub	QKRPSQRSKYL	CAGAAACGTCCGAGCCAGCGTAGCAAATATCTG
13	AMPK	HMRSAMSGLHLVKRR	CATATGCGTAGCGCGATGAGCGGCCTGCATCTGGTGAAACGTCGT
14	autoc	KKALHRQETVDAL	AAAAAAGCGCTGCATCGTCAGGAAACCGTGGATGCGCTG
15	MARCK	KKRFSFKKSFKL	AAAAAACGTTTTAGCTTTAAAAAAAGCTTTAAACTG
16	elong	RAGETRFTDTRKDE	CGTGCGGGCGAAACCCGTTTTACCGATACCCGTAAAGATGAA
17	casei	RRRADDSDDDDD	CGTCGTCGTGCGGATGATAGCGATGATGATGATGAT
18	Cycli	EGVPSTAIREISLLKE	GAAGGCGTGCCGAGCACCGCGATTCGTGAAATTAGCCTGCTGAAAGAA
19	histo	RKRSRLE	CGTAAACGTAGCCGTCTGGAA
20	Ras-G	GKEIYNTIR	GGCAAAGAAATTTATAACACCATTCGT
21	paxII	EETPYSYPTGNHTYQEIA	GAAGAAACCCCGTATAGCTATCCGACCGGCAACCATACCTATCAGGAAATTGCG
22	CDK5	PKTPKKAKKL	CCGAAAACCCCGAAAAAAGCGAAAAAACTG
23	MAPK	KEAEAVTSPR	AAAGAAGCGGAAGCGGTGACCAGCCCGCGT
24	p60c-	YIYGSFK	TATATTTATGGCAGCTTTAAA
25	AKT1/1	GRPRTSSFAEG	GGCCGTCCGCGTACCAGCAGCTTTGCGGAAGGC
26	AKT1/2	RPRAATF	CGTCCGCGTGCGGCGACCTTT
27	CaMKII	KKLNRTLSVA	AAAAAACTGAACCGTACCCTGAGCGTGGCG
28	CHK	KKKVSRSGLYRSPSMPENLNRPR	AAAAAAAAAGTGAGCCGTAGCGGCCTGTATCGTAGCCCGAGCATGCCGGAAAACCTGAACCGTCCGCGT
29	FYN	KVEKIGEGTYGVVYK	AAAGTGGAAAAAATTGGCGAAGGCACCTATGGCGTGGTGTATAAA
30	PKC-S	FKKSFKL	TTTAAAAAAAGCTTTAAACTG
31	p70S61	KKRNRTLTK	AAAAAACGTAACCGTACCCTGACCAAA
32	p70S62	RRRLSSLRA	CGTCGTCGTCTGAGCAGCCTGCGTGCG
33	rerti	NIYISPLKSPYKI	AACATTTATATTAGCCCGCTGAAAAGCCCGTATAAAATT
34	Ca2+/	MHRQETVDCLK	ATGCATCGTCAGGAAACCGTGGATTGCCTGAAA
35	cell2	VRTYTHEVV	GTGCGTACCTATACCCATGAAGTGGTG
36	synti	PLARTLSVAGLPGKK	CCGCTGGCGCGTACCCTGAGCGTGGCGGGCCTGCCGGGCAAAAAA
37	EGF-r	RREELQDDYEDD	CGTCGTGAAGAACTGCAGGATGATTATGAAGATGAT
38	MEK1	QLIDSMANSFVGT	CAGCTGATTGATAGCATGGCGAACAGCTTTGTGGGCACC
39	Abl	EAIYAAPFAKKK	GAAGCGATTTATGCGGCGCCGTTTGCGAAAAAAAAA
40	trans	DLLTSPDVG	GATCTGCTGACCAGCCCGGATGTGGGC
41	cell1	IGEGTYGVV	ATTGGCGAAGGCACCTATGGCGTGGTG
42	malan	RTKRSGSVYEPLKI	CGTACCAAACGTAGCGGCAGCGTGTATGAACCGCTGAAAATT

ly competent Nova Blue *E. coli* cells (Novagen, Schwalbach, Germany) were transformed in parallel with the resulting plasmids according to the protocol provided by Novagen.

2.2. Screening and verification of positive clones

Transformed colonies were analyzed by colony-PCR using external pGEX vector primers and tested for positive expression. Clones containing plasmids with correct reading frames were identified on a Western blot by detecting the C-terminal Streptag of the overexpressed proteins. The identity of expression-positive clones was verified by sequencing.

2.3. Expression of fusion proteins

Overnight cultures of clones harbouring the different expression constructs were grown under vigorous shaking at 200 rpm, 37 °C, in 24-well plates (Whatman, Clifton, NJ, USA) containing 5 ml of LB medium supplemented with 100 μg/ml ampicillin (LB_{Amp}).

These pre-cultures were adjusted with fresh LB_{Amp}

to an $A_{600\ nm} = 0.2$ in a total culture volume of 7.5 ml and incubated at 20 °C with agitation (200 rpm). Gene expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen, Karlsruhe, Germany) to a final concentration of 0.5 m*M* when an $A_{600\ nm}$ of about 0.5 was reached. The incubation was continued overnight and cells were harvested by 10 min centrifugation at 1400 *g*. The resulting cell pellets were stored at −20 °C.

2.4. Lysis and purification

The frozen cell pellets were resuspended in 1 ml lysis-buffer (100 m*M* Tris, pH 8, containing 1 mg/ml lysozyme (Sigma–Aldrich, Steinheim, Germany), 2 μg/ml avidin (Sigma–Aldrich), 10 000-fold diluted benzonase (VWR International, Ismanning, Germany) and protease inhibitors (P 2714, Sigma, Taufkirchen, Germany)) and incubated for 30 min at 37 °C with agitation at 750 rpm. Crude extracts were pre-cleared by centrifugation at 1400 *g* for 10 min to remove cell debris.

The following affinity chromatographic purification procedure was performed by a multichannel pipetting robot (MultiProbe II, Packard, Dreieich, Germany), using its capability for automated liquid aspiration, dispensing and passage of liquids via vacuum filtration.

A specialized 96-well plate (Qiafilter 96 Plate, Qiagen) was used to retain 100 μl Streptactin Macroprep (IBA, Göttingen, Germany) per well. The recombinant proteins were purified on these mini-columns, essentially according to a purification method for Streptag fusion proteins suggested by the manufacturer (IBA). After equilibrating the resin with 1 ml wash-buffer (100 m*M* Tris, pH 8) and packing the Streptactin mini-columns by vacuum filtration, 950 μl of cleared lysates were directly applied to the individual columns. A 30-min incubation step prior to the vacuum application facilitates the binding of Streptag fusion proteins to the resin.

The columns were washed twice with 1 ml of wash-buffer before the three-step elution by competitive displacement with 150 μl of elution-buffer (100 m*M* Tris, pH 8, containing 5 m*M* desthiobiotin (Sigma–Aldrich). Whereas the first elution was carried out with 10 min pre-incubation of elution buffer on the column before vacuum application, for the second and third elution the vacuum was applied immediately.

For a typical filtration step the vacuum was applied for 15 cycles, to guarantee a complete liquid passage in all wells of the resin plate. In one cycle the vacuum of about 800 mbar was switched on for 2 s and switched off for 8 s at the vacuum manifold.

2.5. Regeneration

Regeneration of columns was performed directly in the 96-well resin plate according to a method suggested by the manufacturer of the resin (IBA). Following the wash with 2 ml wash-buffer the remaining desthiobiotin was replaced by application of 2 ml regeneration-buffer (100 m*M* Tris, pH 8, containing 5 m*M* hydroxyazophenylbenzoic acid (Sigma–Aldrich). The organic dye was washed out with 10 ml wash-buffer, whereafter the columns could be used for a new round of purification without loss of capacity or occurrence of cross-contaminations.

2.6. Analytical methods

Protein concentration was measured with a protein assay kit (Roth, Karlsruhe, Germany), using bovine serum albumin (Roth) as a protein standard.

The estimation of protein content, purity and the molecular mass of selected samples was performed with the Protein 200 Plus Assay, according to the protocol provided by the manufacturer (Agilent Technologies, Waldbronn, Germany).

Purified protein and fractions of different experimental steps were analyzed by SDS–PAGE [16] and Western blotting [17]. Proteins were separated on homogeneous 12% polyacrylamide gels under reducing conditions and visualized by staining with colloidal Coomassie Blue (Roth) according to the manufacturer's instructions.

The presence of the Streptag at the C-terminus as indicator for proper full-length expression was analyzed by Western blotting. Proteins were transferred to nitrocellulose membranes and the Streptag of the

fusion proteins was detected by a Streptactin–peroxidase conjugate (IBA).

3. Results

The cloning procedure generated clones containing the proper corresponding sequences for the recombinant peptides as analyzed by colony PCR and sequencing (data not shown).

Expressing clones in 24-well plates according to a standard expression method for medium scale protein expression (induction at $A_{600\ nm}=0.5$ with a final concentration of 0.5 m*M* IPTG and protein production for 4 h at 37 °C) resulted in extraordinarily low yields of the desired proteins (data not shown).

To increase the level of protein production we modified critical expression conditions like culture medium, temperature and expression time. For this purpose we selected one model-clone encoding the protein Autoc (see Table 1) and induced expression in two different media formulations (LB and 2YT) at 37 °C for 4, 5, 6 and 7 h and overnight as well as at 20 °C over night.

The cell pellets were lysed each in 1 ml lysis-buffer, centrifuged for 10 min to sediment cell debris, and the resulting extracts were subsequently used for a test-purification at a vacuum manifold. Cleared lysates and resulting eluates were analyzed with the Protein 200 Plus Assay (Fig. 1). The dominating band within the band spectrum of the 20 °C expression sample (Fig. 1, lane 1) represents the overexpressed protein Autoc with its proper molecular mass of 27 000 g/mol.

All lysates of the 37 °C expression (Fig. 1, lanes 2–5) show two strong bands at 23 000 and 27 000 g/mol corresponding to the full length Autoc and an N-terminally degraded fragment. With prolonged expression time the band with M_r of 27 000 g/mol decreases and the 23 000 g/mol band increases, which indicates the degradation of the recombinant Autoc protein. Degradation at 37 °C (Fig. 1, lanes 2–5) occurs much faster than at 20 °C (Fig. 1, lane 1), where even after an overnight expression no degradation fragment can be detected.

The eluate with the highest yield (600 μg/ml or 180 μg total) and purity (95%) of recombinant Autoc is obtained from the overnight 20 °C expression culture (Fig. 1, lane 6). The 37 °C expression eluates (Fig. 1, lanes 7–10) show lower purity of about 65% caused by co-purification of an N-terminally degraded protein (band at M_r of 23 000 g/mol).

The results of expression in LB-medium were comparable but slightly better than that of expression in 2YT-medium with regard to expression levels or degradation status (data not shown).

As a consequence to the outcome of these tests, all further expression runs, including the expression of the whole set of 42 different clones, were performed at 20 °C overnight in LB-medium.

The Streptag-GST fusion proteins were purified from 7.5 ml of induced culture on 100-μl mini-columns of Streptactin Macroprep. The existing purification protocol for Streptag fusion proteins for HPLC applications (IBA) had to be modified to permit the use of a 96-well plate format on a multichannel pipetting robot.

In contrast to standard column chromatography, where the column packing is a crucial factor, an the case of the mini columns within the 96-well format packing quality is less critical. Since the matrix substance has to withstand extreme conditions, the packing in our case is not that well defined and constant over the purification cycle. Using a vacuum purification device the conditions in the mini columns change with every step. Not only that the resin bed expands and shrinks periodically, but the resin runs dry and becomes equilibrated. Dry-out of the matrix cannot be avoided because a strong vacuum must be applied to guarantee the proper liquid passage through all wells of the matrix plate. In each following liquid dispensing step the resin bed is then again saturated and partially whirled up.

In initial small-scale experiments on the robot we confirmed the tolerance of the chosen Streptactin Macroprep matrix toward such crude procedures, with regard to the resulting purification efficacy.

One limitation of multichannel liquid handling robots is the lack of any online detector unit. The quality of the purification cannot be monitored directly like in HPLC systems, therefore the individual steps of the robot-procedure had to be carefully tested and optimized.

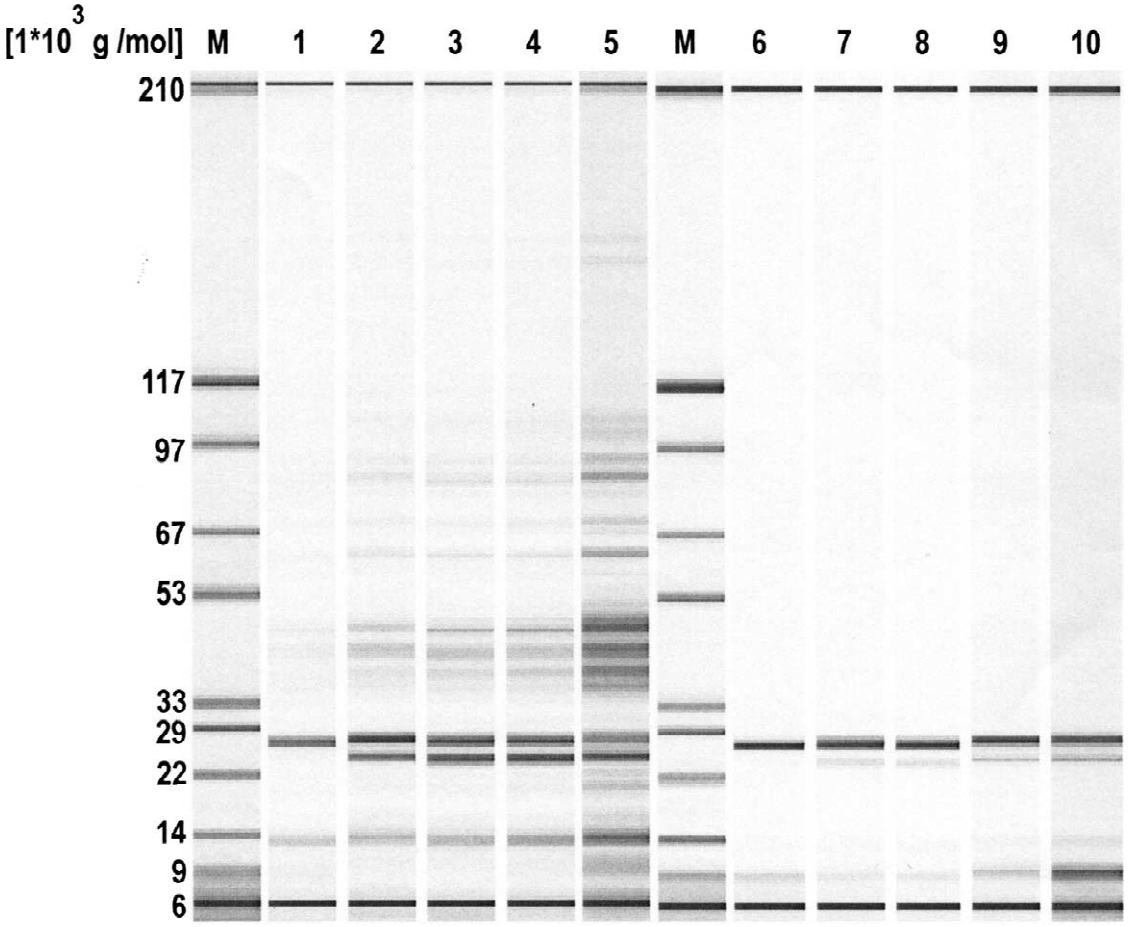


Fig. 1. Analysis of lysates and eluates of expression studies of clone Autoc with the Protein 200 Lab Chip. Lanes 1–5, lysates 1–5 from cultures expressed under different conditions: expressions at 20 °C over night (lane 1), at 37 °C for 4 h (lane 2), 5 h (lane 3), 7 h (lane 4) and over night (lane 5). Lanes 6–10, five eluates corresponding to the purification of lysates 1–5. Four μl of each sample were used for analysis.

The optimization was carried out with cell pellets of our model-clone expressing the protein Autoc. Samples of each purification step were taken and analyzed with the Protein 200 Lab Chip Assay or SDS–PAGE.

In the first optimization step we adjusted the buffer volumes required for the individual chromatographic steps to the new reduced column size. Other modifications of the HPLC method became necessary with regard to the changed retention times of buffer on the column. Liquid passage by vacuum filtration occurs in an extremely short time frame, when compared to flow-rates in HPLC methods. For critical steps of the purification, like binding and elution, when affinity kinetics require a certain retention time, the short contact time between buffer and resin during vacuum filtration was not sufficient. Therefore, additional incubation steps had to be introduced, facilitating binding and elution efficiency. Experiments on the robot showed that the yield of eluted protein could be significantly increased by incubating lysates on the column for 30 min before proceeding with the method. A further increase of yield is obtained with a 10-min incubation time of elution-buffer on the column before applying vacuum for the first elution (data not

shown). Another positive effect we achieved by subdividing the elution step into three successive steps using a minimal volume of 150 μl elution buffer per step. The step by step elution resulted in higher absolute yield of protein than we had reached by eluting with 450 μl in one step (data not shown).

During these experiments clearing of lysates was carried out by vacuum filtration through a 96-well filter plate (Turbofilter 96 Plate, Qiagen). The samples were passed directly into the wells of the 96-well resin plate. Since this led in some cases to frothing and possibly cross-contamination of samples, the filtration clearing step was substituted by centrifugation. Thus foaming and cross-contamination could be avoided successfully.

The final procedure is presented in detail in Fig. 2 and an example of a successful purification is shown in Fig. 3.

Samples of individual experimental steps of the purification of a clone expressing AMPK (see Table 1) are separated on a 12% SDS–Gel. Lysate and flow-through fractions show nearly identical protein patterns. The strong band with a molecular mass of 29 000 g/mol corresponding to the recombinant AMPK protein appears slightly stronger in the lysate (Fig. 3, lane 1) than in the flow-through (Fig. 3, lane 2), which is caused by the binding of a quantity of the fusion protein to the column.

The limited binding capacity of the column in connection with the high-yield expression in the lysate is responsible for the incomplete adsorption of the Streptag fusion protein to the column and its high quantity in the flow-through fraction. In addition, the SDS–PAGE analysis demonstrates that the washing steps were sufficient to remove all non-specifically bound protein (Fig. 3, lane 3). The eluate fractions 1 and 2 (Fig. 3, lanes 4 and 5) contain 95% pure AMPK protein of about 230 μg/ml (data determined with Protein 200 Plus Assay). The lower protein content (70 μg/ml) of the third elution (Fig. 3, lane 6) indicates that the main amount of bound protein was already desorbed from the resin.

Following these proof-of-principle-experiments we demonstrated the lack of cross-contamination while processing 16 different samples in parallel. The arrangement of lysate samples and buffer controls in the 96-well resin plate is illustrated in Fig. 4A. No contamination of buffer controls with any protein was detectable, neither with the protein assay kit nor with SDS–PAGE analysis (Fig. 4B,C). The degradation of eluted protein had already started at the time of sample preparation for the electrophoretic separation, as the protein samples were temporarily stored at 4 °C. Storage at −20 °C immediately after purification will prevent this.

Subsequent to the quantitative and qualitative verification of the robot-procedure we implemented the large-scale protein production of our whole set of kinase substrates within 2 h. Results of the different analysis methods of this process are presented in Fig. 5. Of 42 samples, 36 were obtained at concentrations of 80 up to 590 μg/ml, four samples at concentrations between 30 and 80 μg/ml, one sample could not be purified at all and one, representing the negative GST control, could not be purified, because of its lacking Streptag (Fig. 5E).

The purity of all proteins is estimated from colloidal Coomassie Blue-stained SDS–PAGE (Fig. 5B) to lie in a range of 85 to 90%. Proper identification of the eluted proteins via their C-terminal Streptag was performed by Western blot analysis (Fig. 5D). SDS–PAGE analysis of the 42 lysates (Fig. 5A) shows that protein expression worked well, except for one clone encoding the protein Cell (Fig. 5A, lane 41), which belongs to the samples of lower yield.

We demonstrated the successful processing of 88% of the 42 test proteins for the semi-automated method presented herein, starting with vector constructs of proper sequence.

The advantage of the Streptactin-based affinity chromatography is the possibility of simple and effective matrix regeneration by applying a hydroxyazophenylbenzoic acid containing buffer. We succeeded in regenerating the mini-columns directly within the 96-well resin plate.

With each purification and regeneration cycle the flow resistance of the columns caused by clogging increases and as a result the vacuum application for liquid passages had to be successively prolonged (data not shown).

We performed three complete purification-cycles with Autoc-lysates to investigate the efficiency of the regeneration process. The first purification was carried out under standard conditions on four columns containing clean resin. Subsequent to the first re-

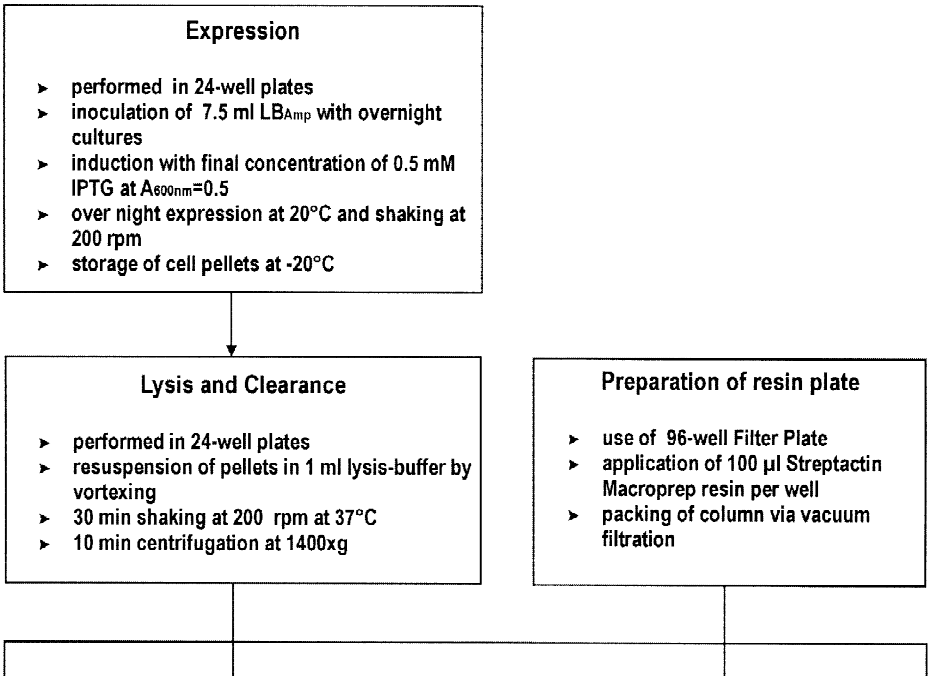
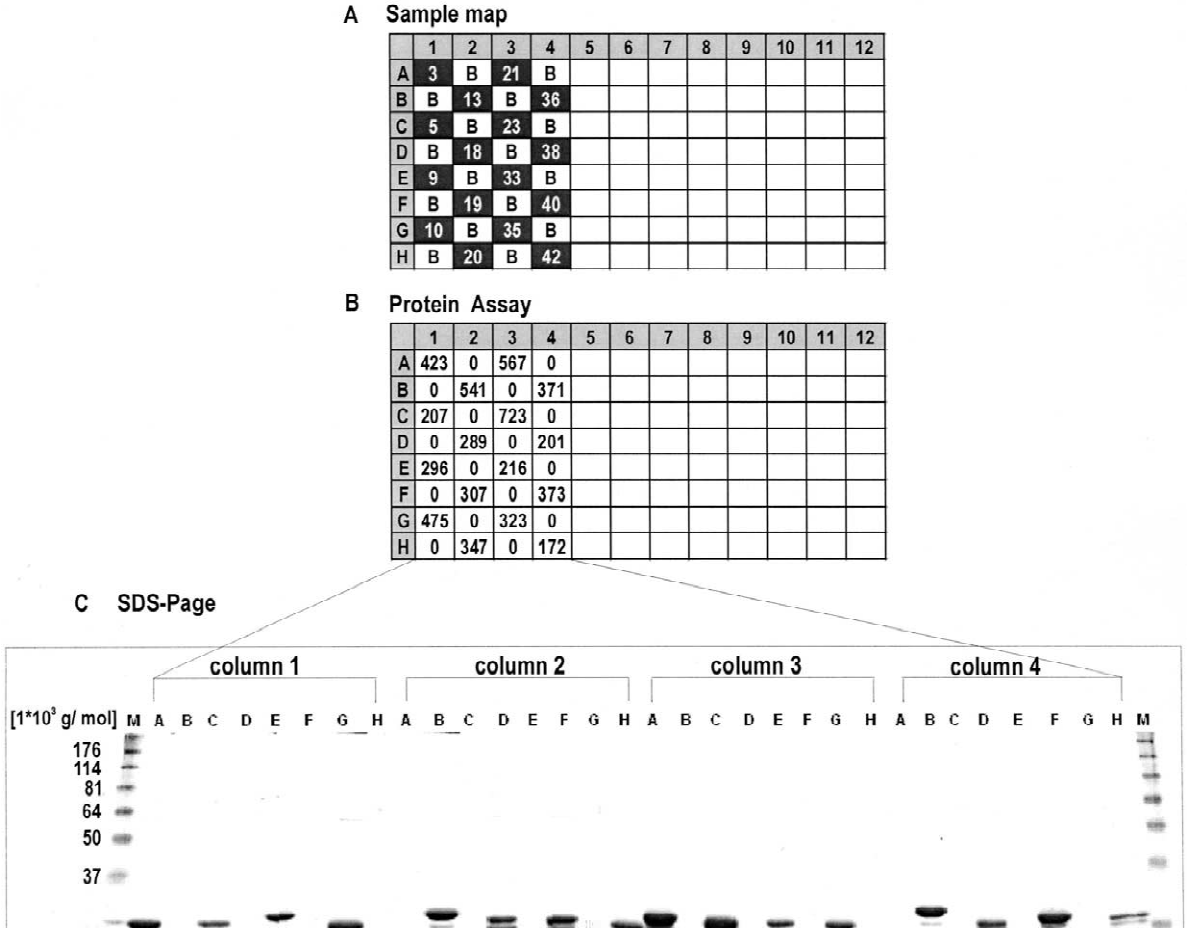


Fig. 2. Flow chart of the protein expression and purification procedure.



A Sample map	1	2	3	4	5	6	7	8	9	10	11	12
A	3	B	21	B								
B	B	13	B	36								
C	5	B	23	B								
D	B	18	B	38								
E	9	B	33	B								
F	B	19	B	40								
G	10	B	35	B								
H	B	20	B	42								

B Protein Assay	1	2	3	4	5	6	7	8	9	10	11	12
A	423	0	567	0								
B	0	541	0	371								
C	207	0	723	0								
D	0	289	0	201								
E	296	0	216	0								
F	0	307	0	373								
G	475	0	323	0								
H	0	347	0	172								

Fig. 4. Analysis of the parallel purification of 16 different Streptag-fusion proteins checking for cross-contamination. (A) Sample map of the arrangement of lysate-samples and buffer-controls in the 96-well resin plate lysates (nomenclature of clones, see Table 1) and buffer controls (B) alternate. (B) Concentrations of eluted proteins obtained by the protein assay kit in μg/ml. (C) SDS–PAGE analysis of eluates. Twelve μl of each sample were applied in the order of the arrangement of the 96-well plate.

Selecting a Streptactin resin for affinity chromatography, we could successfully adapt its very reliable, simple and fast regeneration method to the use in a 96-well plate. This direct processing prevents the loss of any resin in a step wherein it is removed from the plate, while keeping the capacity of the wells constant even after regeneration. Not only does this regeneration procedure reduce costs since the resin can be reused several times, it also saves significant time as a manual emptying or refilling of the plate is unnecessary.

Regeneration can be easily automated and added to the purification process, although the vacuum application for filtration steps has to be successively prolonged for each purification cycle. Since we performed purification/regeneration cycles three times in succession without any noticeable loss of binding capacity of the column, the number of

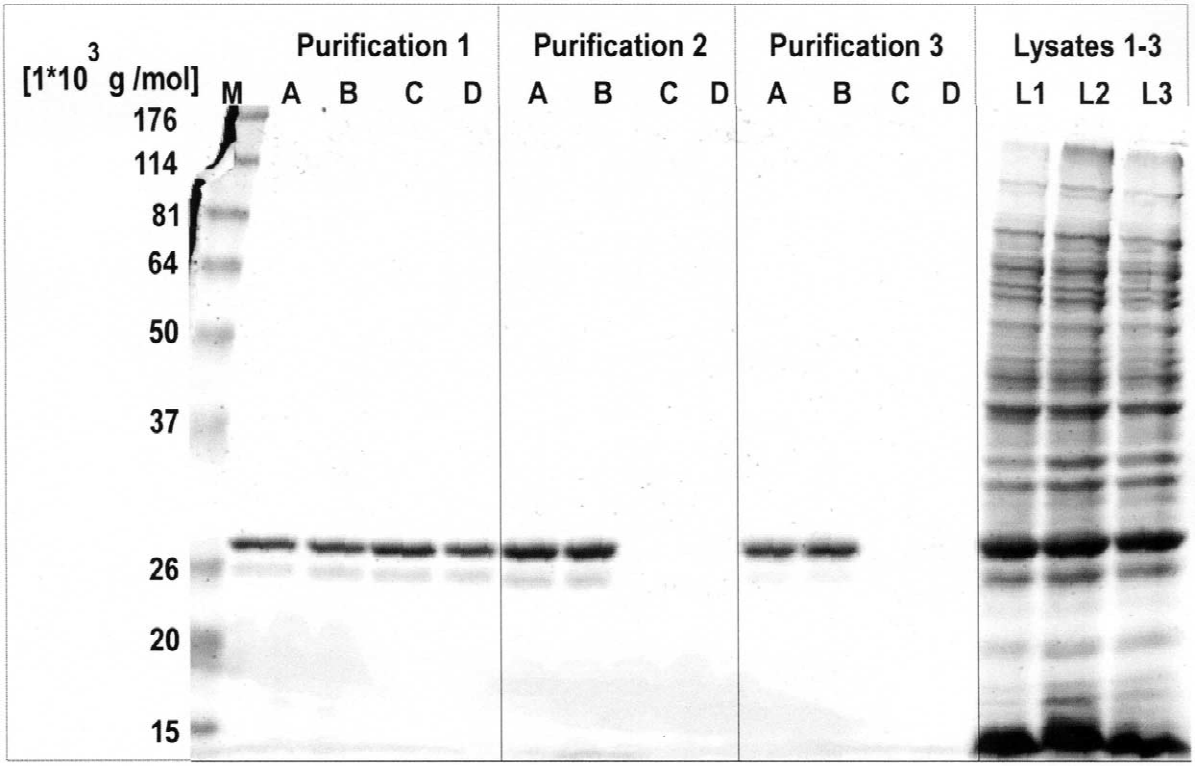


Fig. 6. Analysis of samples obtained from regeneration test. Twelve-μl samples of three successive Autoc-purifications on columns A–D were analyzed by SDS–PAGE. Purification 1: eluates of column A–D, lysate was applied to columns A–D. Purification 2 and 3: eluates of columns A–D, lysate was applied to columns A and B, a buffer-control was applied to columns C and D. Lysates 1–3: lysates (L1–L3) used for purification 1–3.

possible cycles only seems to be limited by increasing column resistance caused by clogging. The exact number of feasible repetitions is yet to be determined.

The above described semi-automated process including cloning, expression and purification/regeneration represents a useful tool in the field of high-throughput protein production, which is one of the key technological tools for the functional analysis of large numbers of proteins in parallel, as for example on protein arrays.

Acknowledgements

We would like to thank Urs Hanke for technical support with the robot and Sandra Mills for helpful suggestions regarding this manuscript.

References

[1] M. Larsson, S. Graslund, L. Yuan, E. Brundell, M. Uhlen, C. Hoog, S. Stahl, J. Biotechnol. 80 (2000) 143.
[2] D. Christendat, A. Yee, A. Dharamsi, Y. Kluger, A. Savchenko, J.R. Cort, V. Booth, C.D. Mackereth, V. Saridakis, I. Ekiel, G. Kozlov, K.L. Maxwell, N. Wu, L.P. McIntosh, K. Gehring, M.A. Kennedy, A.R. Davidson, E.F. Pai, M. Gerstein, A.M. Edwards, C.H. Arrowsmith, Nat. Struct. Biol. 7 (2000) 903.
[3] M.R. Martzen, S.M. McCraith, S.L. Spinelli, F.M. Torres, S. Fields, E.J. Grayhack, E.M. Phizicky, Science 286 (1999) 1153.
[4] J.S. Albala, I. Humphery-Smith, Curr. Opin. Mol. Ther. 1 (1999) 680.
[5] J.S. Albala, K. Franke, I.R. McConnell, K.L. Pak, P.A. Folta, B. Rubinfeld, A.H. Davies, G.G. Lennon, R. Clark, J. Cell Biochem. 80 (2000) 187.
[6] R.K. Knaust, P. Nordlund, Anal. Biochem. 297 (2001) 79.
[7] M. Nasoff, M. Bergseid, J.P. Hoeffler, J.A. Heyman, Methods Enzymol. 328 (2000) 515.

[8] R.C. Stevens, Structure Fold Des. 8 (2000) 177.
[9] S.A. Lesley, Protein Expr. Purif. 22 (2001) 159.
[10] M. Gilbert, J.S. Albala, Curr. Opin. Chem. Biol. 6 (2002) 102.
[11] H. Zhu, J.F. Klemic, S. Chang, P. Bertone, A. Casamayor, K.G. Klemic, D. Smith, M. Gerstein, M.A. Reed, M. Snyder, Nat. Genet. 26 (2000) 283.
[12] H. Zhu, M. Bilgin, R. Bangham, D. Hall, A. Casamayor, P. Bertone, N. Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R.A. Dean, M. Gerstein, M. Snyder, Science 293 (2001) 2101.
[13] D.B. Smith, K.S. Johnson, Gene 67 (1988) 31.
[14] A. Skerra, T.G. Schmidt, Methods Enzymol. 326 (2000) 271.
[15] J. Sambrook, E.F. Fritsch, T. Maniatis, in: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[16] U.K. Laemmli, Nature 227 (1970) 680.
[17] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350.
[18] P. Braun, Y. Hu, B. Shen, A. Halleck, M. Koundinya, E. Harlow, J. LaBaer, Proc. Natl. Acad. Sci. USA 99 (2002) 2654.
[19] R. Felleisen, V. Zimmermann, B. Gottstein, N. Muller, Biotechniques 20 (1996) 616.
[20] C. Draveling, L. Ren, P. Haney, D. Zeisse, M.W. Qoronfleh, Protein Expr. Purif. 22 (2001) 359.
[21] T. Lanio, A. Jeltsch, A. Pingoud, Biotechniques 29 (2000) 338.