November 2005

EasyXpress™ Protein Synthesis Handbook

EasyXpress Linear Template Kit Plus

EasyXpress pIX3.0 Vector

EasyXpress Protein Synthesis Mini Kit

EasyXpress Protein Synthesis Maxi Kit

EasyXpress Random Biotin Kit

For scalable in vitro synthesis of recombinant proteins and generation of linear expression templates by PCR

QIAGEN

Contents

Kit Contents

EasyXpress Linear Template Kit Plus (20)	
Cat. no.	**32723**
Number of reactions	**20**
ProofStart® DNA Polymerase (orange screw-cap)	40 µl (100 U)
10x ProofStart PCR Buffer (blue screw-cap)	1 ml
25 mM $MgSO_4$ (yellow screw-cap)	1 ml
dNTP Mix (10 mM each) (purple screw-cap)	200 µl
5x Q-Solution (green screw-cap)	400 µl
RNase-Free Water (colorless screw-cap)	1 x 1.9 ml
EasyXpress Positive-Control DNA (PCR) (white screw-cap)	1 x 10 µl
Positive-Control Sense Primer (white screw-cap)	15 µl
Positive-Control Antisense Primer (white screw-cap)	15 µl
Strep-tag™ Sense Primer (yellow screw-cap)	40 µl
Strep-tag Antisense Primer (brown screw-cap)	40 µl
6xHis tag Sense Primer (yellow screw-cap)	40 µl
6xHis tag Antisense Primer (brown screw-cap)	40 µl
No tag Sense Primer (yellow screw-cap)	40 µl
No tag Antisense Primer (brown screw-cap)	40 µl
XE-Solution (green screw-cap)	40 µl
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EasyXpress pIX3.0 Vector	**Cat. no. 32733**
EasyXpress pIX3.0 Vector (white screw-cap)	25 µg (0.5 µg/µl)
Handbook	1

EasyXpress Protein Synthesis Kit	Mini Kit	Maxi Kit
Number of reactions	**20 x 50 µl reactions**	**4 x 1 ml reactions**
Cat. no.	**32502**	**32506**
EasyXpress *E. coli* Extract (colorless snap-cap)	20 x 17.5 µl	4 x 350 µl
EasyXpress Reaction Buffer (blue screw-cap)	1 x 400 µl	4 x 450 µl
RNase-Free Water (colorless screw-cap)	1 x 1.9 ml	1 x 1.9 ml
EasyXpress Positive-Control DNA (yellow screw-cap)	1 x 50 µl	4 x 50 µl
Handbook	1	1

EasyXpress Random Biotin Kit	
Cat. no.	**32612**
Number of reactions	**20 x 50 µl reactions**
EasyXpress *E. coli* Extract (colorless snap-cap)	20 x 17.5 µl
EasyXpress Reaction Buffer (blue screw-cap)	1 x 400 µl
RNase-Free Water (colorless screw-cap)	1 x 1.9 ml
EasyXpress Positive-Control DNA (yellow screw-cap)	1 x 10 µl
EasyXpress Biotinyl-Lysyl tRNA (Phe) (orange screw-cap)	4 x 15 µl
Handbook	1

Shipping and Storage

The **EasyXpress Linear Template Kit Plus** is shipped on dry ice and should be stored immediately upon receipt at –20°C in a constant-temperature freezer.

The **EasyXpress pIX3.0 Vector** is shipped on dry ice and must be stored at –20°C upon arrival.

EasyXpress Protein Synthesis Mini and Maxi Kits are shipped on dry ice. All components must be stored at –70°C. Once thawed, *E. coli* extract should be stored on ice and used within 4 hours. Do not refreeze and thaw more than two times. Refreeze the extract in liquid nitrogen.

The EasyXpress Random Biotin Kit is shipped on dry ice. All components must be stored at –70°C. Once thawed, EasyXpress *E. coli* Extract should be stored on ice and used within 4 hours. Do not thaw and refreeze more than four times. Once thawed, Biotinyl-Lysyl tRNA should be stored on ice and quickly returned to a –70°C freezer after use. Do not refreeze and thaw more than two times. Refreeze the extract in liquid nitrogen.

When stored under the above conditions and handled correctly, all kits can be kept for at least 6 months without showing any reduction in performance. The EasyXpress pIX3.0 Vector can be stored for at least 1 year.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany, Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of EasyXpress Kits is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

EasyXpress kits are developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside back cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN® products. If you have any questions or experience any difficulties regarding EasyXpress kits or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see inside back cover).

Introduction

Proteins such as enzymes, antibodies, hormones, and structural elements play essential roles in nearly all biological processes. Therefore, great efforts have been made to develop technologies for the production of proteins using recombinant technology. Using modern protein engineering methods, which include cloning of DNA sequences and the in vivo expression of genes, it is possible to produce specific proteins in large amounts and also produce proteins with improved or altered biological activities.

Several factors must be carefully considered when producing recombinant proteins using in vivo expression methods. Cells must be transformed with an expression construct (e.g., plasmid DNA), and transformants containing the correct construct must be selected and cultivated. Overexpression of proteins that are toxic to the host cells can be difficult. Cell lysis and procedures used for purification of protein from whole cell lysates can be complicated; problems may arise because of aggregation or degradation of proteins within the cell.

In most cases these limitations can be overcome by the use of cell-free protein biosynthesis systems (also termed in vitro translation systems), which are often seen as a very attractive alternative to classical in vivo expression systems. In vitro translation generates proteins by coupled or successive transcription and translation in cell-free extracts of prokaryotic or eukaryotic cells. The advantages of in vitro translation systems include time savings, the possibility to produce proteins that are toxic or have modified or isotope-labeled amino acids, a high protein yield per unit volume, and the ability to adapt reaction conditions to the requirements of the synthesized protein (for example, the inclusion of protease inhibitors). Compared with current cloning techniques, another important advantage offered by an in vitro translation system is the possibility of using PCR-product templates for protein synthesis. This greatly accelerates the protein production process, because no cloning steps are required.

Proteins produced by in vitro translation can be used for a wide variety of downstream applications, including activity assays, structural and mutational analyses, protein–protein interaction studies, and the expression and analysis of open reading frames.

The EasyXpress System

QIAGEN has developed the EasyXpress system to be the easiest and fastest way to produce recombinant protein. The fully integrated system offers solutions for all scales of protein production, from micrograms of protein for initial analyses to milligram amounts for comprehensive structural and functional studies. The EasyXpress system dovetails seamlessly with other high-quality QIAGEN® products for protein science (such as Ni-NTA resins, which set the standard for purification of 6xHis-tagged proteins), and forms an integral part of QIAGEN's comprehensive and ever-expanding protein portfolio (see Figure 1).

Using the EasyXpress Linear Template Kit Plus, researchers can quickly determine the optimal makeup of an expression construct. Once this construct has been identified, it can be easily cloned into the pIX3.0 vector and used to express proteins in large quantities with EasyXpress Mega or NMR kits, or used to produce biotin-labeled proteins with the EasyXpress Random or Site-Specific Biotin Kits. Alternatively, the cloned construct can be used to transform *E. coli* cells for in vivo expression. To learn more about the EasyXpress system, visit www.qiagen.com/easyxpress .

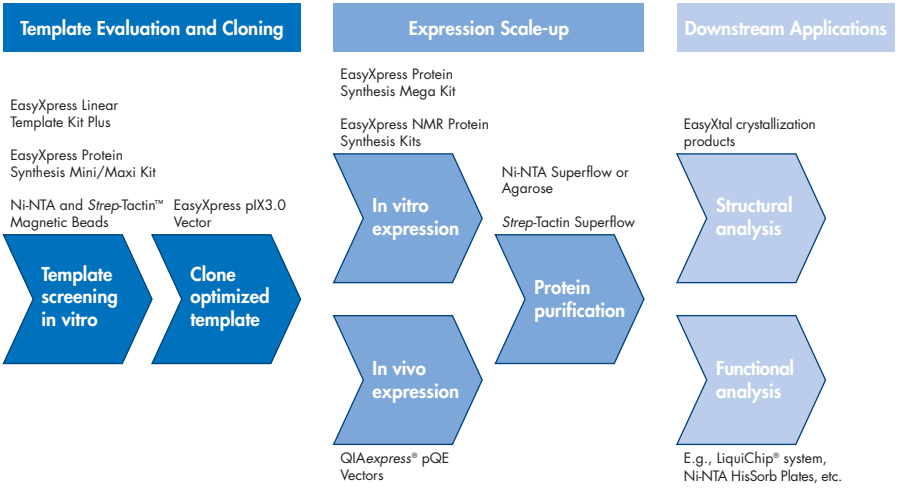

Figure 1 Expression, purification, detection, and analysis form an integrated workflow chain.

Principle and Procedure

E. coli-based EasyXpress Protein Synthesis Kits use highly productive *E. coli* cell lysates, which contain all translational machinery components (ribosomes, ribosomal factors, tRNAs, aminoacyl-tRNA synthetases, etc.) as well as T7 RNA polymerase. They use a coupled transcription–translation system that can be used to express full-length proteins from T7 or *E. coli* promoters in a single-step reaction using plasmid or linear DNA templates, such as PCR products (Figure 2). Using EasyXpress Protein Synthesis Mini or Maxi Kits, up to 600 µg/ml biologically active protein can be synthesized within 1 hour. The synthesis reaction can be easily scaled up (to produce milligram amounts of protein) or down (for high-throughput screening in 96-well plate format). The amount of protein synthesized increases linearly with reaction volume.

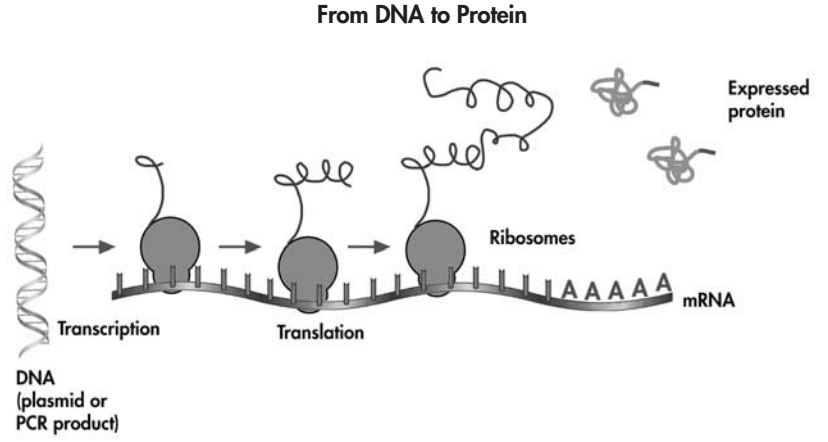

Figure 2 Schematic showing in vitro translation of proteins.

The EasyXpress Linear Template Kit Plus uses a two-step PCR process to generate linear DNA templates for in vitro translation systems. Using specially designed primers, coding DNA sequence is both amplified and supplemented with regulatory elements required for optimal transcription and translation in cell-free expression systems. Specially designed 5' untranslated regions (UTRs) on the sense adapter primer sequences reduce the formation of secondary structure in the translation initiation region, one of the commonest causes of low expression rates. A His-or *Strep*-tag II can be added to either terminus, greatly simplifying protein purification and detection. The N-terminal *Strep*-tag construct also contains a Factor Xa Protease cleavage site, for easy tag removal. The fast procedure enables researchers to discover the optimal template structure within a single working day (Figure 3).

The EasyXpress pIX3.0 vector is designed for cloning of PCR products generated by the EasyXpress Linear Template Kit Plus. The PCR products contain multiple cloning sites that are compatible with the multiple cloning site of the pIX3.0 vector (see Appendix B, page 50).

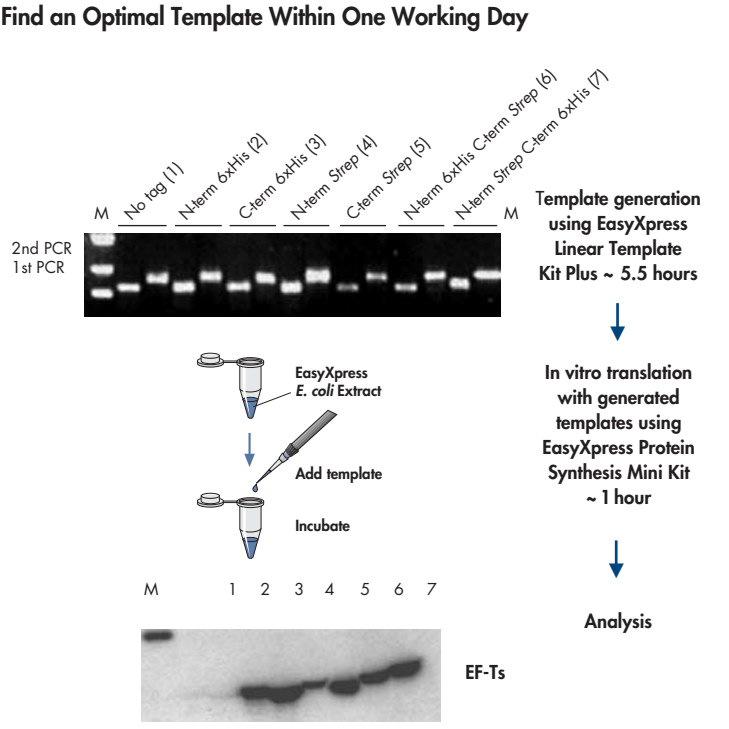

Figure 3 Identification of the optimal expression construct maximizes yields in large-scale in vitro reactions or in vivo expression. The western blot was probed with anti-His- and *Strep*-tag antibodies. Therefore, the untagged protein is not detected.

Expression Templates

EasyXpress Kits can be used to express proteins from a variety of DNA templates as long as they contain a T7 or a strong *E. coli* promoter upstream from the coding sequence and a ribosome binding site. Suitable DNA templates include supercoiled plasmids and linear DNA (e.g., PCR products).

Plasmid DNA

T7 promoter based constructs, including the pET plasmid series (Novagen), are a suitable basis for generating expression constructs. Such vectors provide mRNA-stabilizing secondary structures in the 5' and 3' untranslated regions (UTRs) that play an important role in increasing the efficiency of expression. Use of vectors is not restricted to T7 promoter based constructs. QIAGEN's pQE vectors have been used successfully in these applications. Table 1 gives an overview of which vectors have successfully been used with EasyXpress Kits. Greatest yields of protein are obtained using template DNA of the highest purity. High-purity plasmid DNA can easily be obtained with the QIAGEN HiSpeed®, QIAfilter, and QIAprep® Kits. DNA prepared using the standard alkaline lysis method described by Sambrook, Fritsch, and Maniatis (1) may be sufficiently pure, but DNA must be free of RNases. The concentration of plasmid DNA in each 50 µl in vitro translation reaction should be 5–10 nM, which corresponds to 0.5–1 µg for plasmids up to 5 kb in size, or 1–2 µg for plasmids >5 kb. For plasmids containing an *E. coli* promoter, the higher concentration of 10 nM should be used.

pIX3.0 Vector

The EasyXpress pIX3.0 Vector (see Appendix B) has been developed to enable easy cloning of PCR products generated using the EasyXpress Linear Template Kit Plus. Its multiple cloning site (MCS) is compatible with restriction sites in the sense and antisense adapter primers supplied with the EasyXpress Linear Template Kit Plus (see Figure 7, page 19). Once cloned into pIX3.0, expression constructs can be used to generate larger amounts of protein in large-scale protein synthesis reactions. Alternatively, the cloned epxression construct can be used to transform *E. coli* cells for conventional in vivo expression.

Table 1. Successfully Tested Vectors for EasyXpress Synthesis Reactions

Vector	Promoter	Protein
pIX3.0	T7 (from PCR product)	EF-Ts
pET15b	T7	Cytohesin-1/SEC7*
pET43	T7	NusA
pET3d	T7	Several different proteins
TAGZyme™ pQE-2	T5	TNFα
pQE-30	T5	Thioredoxin
pIVEX series	T7	GFP

* Expression construct kindly provided by Michael Blind, NascaCell IP GmbH (Germany).

PCR products

If PCR products are added to in vitro translation reactions, we recommend that they are generated using the EasyXpress Linear Template Kit Plus (cat. no. 32723). PCR products can be added directly to in vitro translation reactions without further cleanup. The amount of PCR product added to each 50 µl in vitro translation reaction should be 0.7–1.0 µg.

If PCR products need to be concentrated, we recommend using the QIAGEN MinElute® PCR Purification Kit. If using PCR products as a template, XE-Solution provided with the EasyXpress Linear Template Kit Plus should be added to in vitro translation reactions to increase the efficiency of transcription and translation. The adapter primers supplied with the EasyXpress Linear Template Kit Plus are compatible with XE-Solution.

The section "Generating PCR Products for In Vitro Translation" on pages 19–23 gives comprehensive and detailed information on producing PCR products suitable for use as expression constructs with EasyXpress Protein Synthesis Kits.

Purification of In Vitro-Synthesized Proteins

For many applications where labeled proteins are used (e.g., BIACORE® and *x*MAP™ or microarray studies), purification is not necessarily required. Also, detection of randomly labeled protein (e.g., by western blot analysis), does not require prior purification. However, there may be applications where the protein background from the *E. coli* lysate may interfere with downstream assays. Proteins carrying a 6xHis or *Strep*-tag can be affinity purified in a fast one-step procedure using Ni-NTA or *Strep*-Tactin matrices (see Figure 4).

To ensure that only full length protein is purified, proteins that are known to easily fragment should be purified using C-terminal affinity tags.

Purification of 6xHis-tagged proteins using Ni-NTA matrices

Purification of 6xHis-tagged proteins is based on the remarkable selectivity and high affinity of patented Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues — the 6xHis tag.

NTA, which has four chelation sites for nickel ions, binds nickel more tightly than metal-chelating purification systems that only have three sites available for interaction with metal ions. The extra chelation site prevents nickel-ion leaching: providing a greater binding capacity, and high-purity protein preparations.

In vitro-synthesized proteins that carry a 6xHis tag can be easily purified using Ni-NTA Magnetic Agarose Beads or Ni-NTA Superflow. For purification protocols, see Appendix A, page 44.

Purification of *Strep*-tagged proteins using *Strep*-Tactin

The *Strep*-tag II is a short peptide (8 amino acids, WSHPQFEK), which binds with high selectivity to *Strep*-Tactin, an engineered streptavidin. The binding affinity of the *Strep*-tag II to *Strep*-Tactin (K_d = 1 µM) is nearly 100 times higher than to streptavidin. After a short washing step, gentle elution of purified recombinant protein is performed by addition of low concentrations of biotin or desthiobiotin. Desthiobiotin is a stable, reversibly binding analog of biotin, the natural ligand of streptavidin.

The N-terminal *Strep*-tag Sense Primer also contains a Factor Xa Protease cleavage site, for easy tag removal (see primer sequence, page 50).

Note: It is not advisable to purify biotinylated, *Strep*-tagged proteins using *Strep*-Tactin matrices, as the biotin moiety will bind with extremely high affinity, effectively immobilizing the protein.

Micro-scale Purification of His- and *Strep*-tagged Proteins Expressed Using the EasyXpress Protein Synthesis Kit

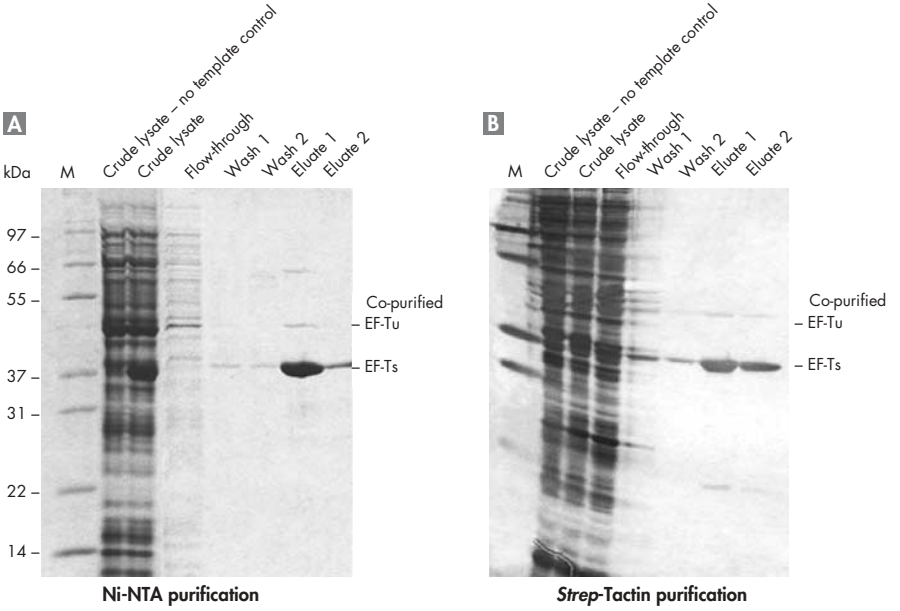

Figure 4 A 6xHis-tagged or B *Strep*-tagged *E. coli* elongation factor-Ts was purified under native conditions from 50 µl EasyXpress Protein Synthesis Mini Kit in vitro translation reactions. Ni-NTA Magnetic Agarose Bead or *Strep*-Tactin Magnetic Bead suspension (150 µl) was added to the crude lysate to bind protein. The beads were washed with 500 µl wash buffer to remove contaminants and pure 6xHis- or *Strep*-tagged protein was eluted in two 50 µl aliquots of elution buffer. Protein from each fraction was separated by SDS-PAGE and visualized by A Coomassie® or B silver staining. In both cases, EF-Ts is eluted from Ni-NTA or *Strep*-Tactin beads as a complex with endogenous elongation factor EF-Tu demonstrating the functional activity of His- and *Strep*-tagged EF-Ts synthesized in vitro using EasyXpress *E. coli* extract. **M**: markers.

Producing Biotin-Labeled Proteins

Labeling proteins can dramatically simplify their study, provided that the labeling procedure does not influence protein structure or function. Adding a specific label or tag to different proteins enables their immobilization or detection using a common principle.

For small-scale analyses, synthesized proteins are usually visualized by detection of radioactively labeled amino acids incorporated during translation. However, incorporating radioactively labeled amino acids — such as [^{35}S] methionine or [^{14}C] leucine — is time-consuming, generates hazardous waste, and requires extra safety precautions. There is therefore a need for alternative, non-radioactive methods for detecting in vitro translated proteins.

QIAGEN offers the EasyXpress Random Biotin Kit for random co-translational non-radioactive labeling of proteins. Using in vitro translation in *E. coli* extracts, recombinant biotinylated proteins are produced in high yields (see protocols on pages 29 and 33). The biotin moiety greatly facilitates detection of any recombinant protein using a universally applicable method. The EasyXpress Random Biotin Kit is superior to comparable solutions with respect to ease-of-use and detection sensitivity.

Random biotin labeling

The EasyXpress Random Biotin Kit uses a synthetic aminoacylated tRNA that carries a biotinylated lysine residue and the phenylalanine anticodon GAA for co-translational labeling of recombinant proteins (Figure 5). To incorporate biotin into a recombinant protein, a standard EasyXpress protein synthesis reaction is performed in the presence of the biotinyl-lysyl tRNA (see Table 2). The biotinyl-lysyl tRNA recognizes the Phe codon on the mRNA strand and during protein synthesis either biotin-labeled lysine or the respective unlabeled amino acid (from the reaction buffer) are incorporated. Biotin incorporation occurs statistically, but with high efficiency (Figure 6). This represents a universal, easy-to-use labeling method that enables high-sensitivity detection of recombinant proteins, for example using streptavidin conjugates following western blotting.

For applications such as protein-protein interaction analysis using the LiquiChip®, other xMAP, or BIACORE systems and for directed immobilization we recommend using the EasyXpress Site-Specific Biotin Kit (QIAGEN cat.no. 32602), which allows site-specific incorporation of biotin at a stoichiometry of 1: 1 (i.e., one biotin molecule per protein molecule).

Table 2. Biotinyl-Lysyl tRNA in the EasyXpress Random Biotin Kit

tRNA	tRNA anticodon	mRNA codon	Replaced amino acid	Incorporated amino acid
Bio-Lys tRNA (Phe)	GAA	UUC	Phenylalanine	Biotinyl-lysine

Random Biotin Labeling Using the EasyXpress Random Biotin Kit

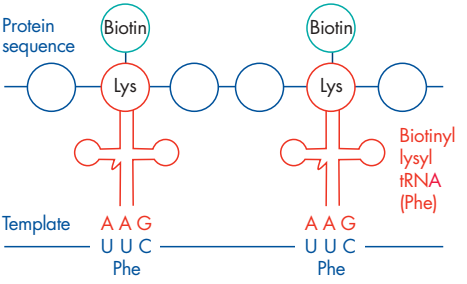

Figure 5 Schematic representation of biotin incorporation using the EasyXpress Random Biotin Kit.

Efficient Incorporation of Biotin into Synthesized Proteins

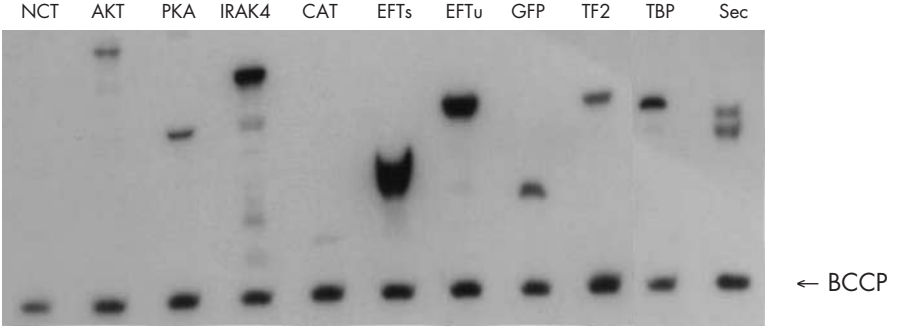

Figure 6 Synthesis of biotinylated recombinant proteins using the QIAGEN EasyXpress Random Biotin Kit, which inserts biotinylated lysine residues at phenylalanine UUC codons. **BCCP**: endogenous *E. coli* biotin carboxyl carrier protein; **NTC**: no template control; **AKT**: human RAC-alpha serine/threonine kinase; **PKA**: human cAMP-dependent protein kinase, beta catalytic subunit; **IRAK4**: human interleukin-1 receptor associated kinase 4; **CAT**: chloramphenicol acetyltransferase; **EFTs**: elongation factor Ts; **EFTu**: elongation factor Tu; **GFP**: green fluorescent protein; **TF2**: human Transcription Factor IIB; **TBP**: human TATA-box binding protein; **SEC7**: human cytohesin-1 SEC7phs domain.

Generating PCR Products for Use in In Vitro Translation Reactions

The EasyXpress Linear Template Kit Plus uses a two-step procedure to generate PCR products suitable for in vitro translation in prokaryotic-cell extracts. In the first step, defined 5'-tails are added to PCR products using gene-specific primers. The 5'-tails serve as hybridization sites for primers used in a second PCR, in which DNA is amplified using adapter primers that code for regulatory elements required for optimal expression in prokaryotic-cell extracts. These elements include a T7 promoter, ribosomal binding site, and T7 terminator. The resulting PCR products contain multiple cloning sites that are compatible for cloning into the EasyXpress Vector pIX3.0 (Figure 7).

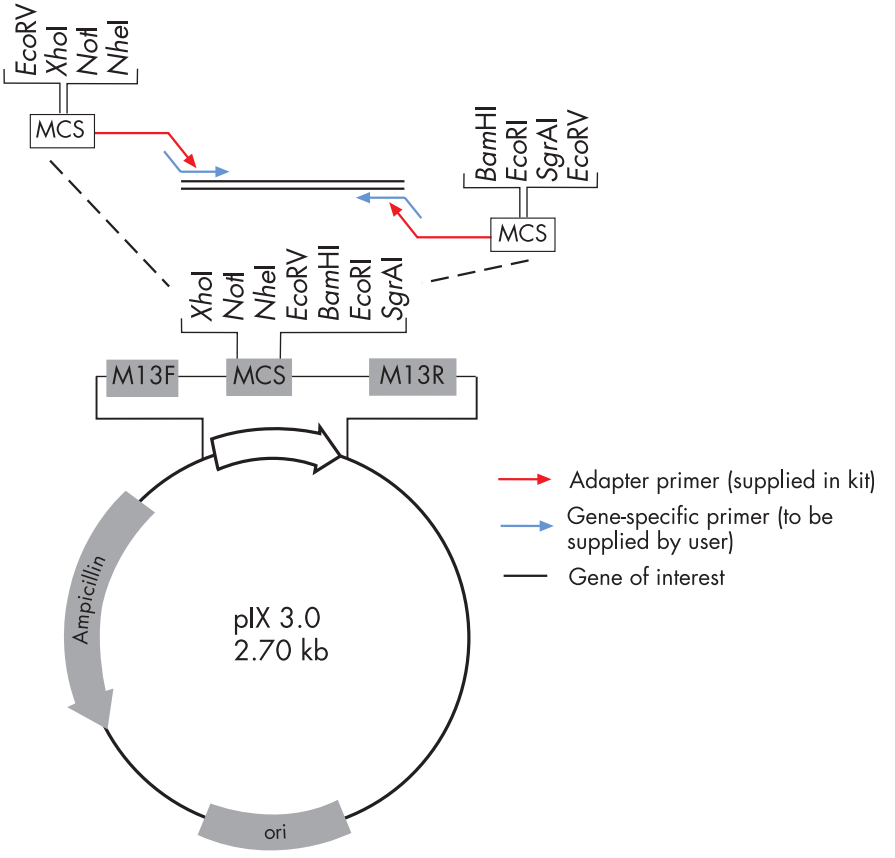

Figure 7 Straightforward cloning into vector pIX3.0 for expression scale-up.

Adapter primers that encode N- or C-terminal affinity tags are contained in the kit. Addition of affinity tags to constructs greatly facilitates purification and detection of expressed proteins. Different combinations of adapter primers can be used to generate singly or doubly tagged proteins with a His- or *Strep*-tag at either terminus (Figure 3). In addition to the *Strep*-tag II epitope, the N-Terminal *Strep*-tag adapter primer encodes for a Factor Xa Protease Cleavage site between the tag and the body of the target protein.

In addition to adapter primers, the EasyXpress Linear Template Kit Plus also provides ProofStart DNA Polymerase. The combination of ProofStart DNA Polymerase — a unique hot-start high-fidelity enzyme — and ProofStart PCR Buffer minimizes the need for PCR optimization and optimizes PCR product yield. The kit contains enough reagents for 20 two-step PCRs, with each reaction yielding enough expression template for 3–4 in vitro translation reactions. The final PCR product can be added to the in vitro translation reaction without any further purification steps. The user must provide a DNA template encoding the protein of interest, and two gene-specific PCR primers.

Plasmid DNA, genomic DNA mixtures, or cDNA mixtures can be used as a template for the first PCR. Alternatively, cDNA can be generated by reverse transcription PCR (RT-PCR) using a gene-specific antisense primer (with defined 5' tail sequence, see Table 3, page 22), total RNA, and a reverse transcriptase. QIAGEN offers Omniscript® and Sensiscript® RT Kits for efficient RT-PCR. Rules for the design of the gene-specific PCR primers containing the relevant 5' tails are given on page 22.

For optimal expression using PCR products in in vitro translation reactions, XE-Solution is provided. XE-Solution is added to in vitro translation reactions where it protects linear DNA from degradation by exonucleolytic nucleases.

Strategy for designing gene-specific primers

Prerequisites for successful PCR include the design of optimal gene-specific primer pairs, the use of appropriate primer concentrations, and the correct storage of primer solutions. Primers (which, for best results, should be HPLC-purified) can be ordered from Operon Biotechnologies at www.operon.com .

The final PCR product added to the in vitro translation reaction is generated by a two-step PCR procedure. In the first PCR, primers must be designed that are not only specific for the protein of interest, but also provide 5' tails that will act as hybridization sites for adapter primers used in the second PCR. The first step in designing primers is to decide whether an affinity tag should be attached to the protein and at which terminus. Use the 5'-end sequence information in Table 3 and the information below to design forward (sense) and reverse (antisense) primers for protein constructs with an affinity tag at the respective terminus.

Length

- The length of the gene-specific sequence should be 17–20 bases (see Table 3). This may be reduced or increased in some cases to give primers suitable for the annealing temperature of 50°C.

Melting temperature (*Tm*)

- The optimal melting temperature (*Tm*) for primers used with the EasyXpress Linear Template Kit Plus is 55°C. The optimal annealing temperature is 5°C below *Tm*.

  Simplified formula for estimating melting temperature (*Tm*):

  Tm = 2°C x (A+T) + 4°C x (G+C)

  Whenever possible, design primer pairs with similar *Tm* values.

Table 3. Sequences of Gene-Specific Primers Required for First-Round PCR.

Desired feature(s)	Gene-specific sense primer	Gene-specific antisense primer
No tag	5'-AGAAGGAGATAAACA + **ATG** + 17 nt target sequence (ATG =start codon)	5'-CTTGGTTAGTTAGTTA + **TTA** + 20 nt target sequence (TTA = stop codon)
N-terminal 6xHis tag	5'-ACC CAC GCG CAT GTC GTA AAA AGC ACC CAA + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-CTTGGTTAGTTAGTTA + **TTA** + 20 nt target sequence (TTA = stop codon)
C-terminal 6xHis tag	5'-AGAAGGAGATAAACA + **ATG** + 17 nt target sequence (ATG =start codon)	5'-TG GTG ATG GTG GTG ACC CCA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
N-terminal *Strep*-tag	5'-AAA AGC GCT GAA AAC CTG ATC GAA GGC CGT + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-CTTGGTTAGTTAGTTA + **TTA** + 20 nt target sequence (TTA = stop codon)
C-terminal *Strep*-tag	5'-AGAAGGAGATAAACA + **ATG** + 17 nt target sequence (ATG =start codon)	5'-GG ATG AGA CCA GGC AGA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
N-terminal 6xHis tag and C-terminal *Strep*-tag	5'-ACC CAC GCG CAT GTC GTA AAA AGC ACC CAA + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-GG ATG AGA CCA GGC AGA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
N-terminal *Strep*-tag and C-terminal 6xHis tag	5'-AAA AGC GCT GAA AAC CTG ATC GAA GGC CGT + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-TG GTG ATG GTG GTG ACC CCA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)

Table 4. Sense and Antisense Primer Pairs Required for Second-Round PCR.

Desired feature(s)	N-terminus sense primer* (yellow screw-cap)	C-terminus antisense primer* (brown screw-cap)
No tag	No tag Sense Primer	No tag Antisense Primer
N-terminal 6xHis tag	6xHis tag Sense Primer	No tag Antisense Primer
C-terminal 6xHis tag	No tag Sense Primer	6xHis tag Antisense Primer
N-terminal *Strep*-tag	*Strep*-tag Sense Primer†	No tag Antisense Primer
C-terminal *Strep*-tag	No tag Sense Primer	*Strep*-tag Antisense Primer
N-terminal 6xHis tag and C-terminal *Strep*-tag	6xHis tag Sense Primer	*Strep*-tag Antisense Primer
N-terminal *Strep*-tag and C-terminal 6xHis tag	*Strep*-tag Sense Primer†	6xHis tag Antisense Primer

* Nucleotide sequences of primers can be found in Appendix B, page 50.

† In addition to the *Strep*-tag II epitope, the *Strep*-tag Sense Primer encodes a Factor Xa Protease Cleavage site between the tag and the body of the target protein.

Positive control for the two-step PCR procedure

The functionality of the kit and the PCR procedure is checked by performing a two-step positive-control PCR. The first positive-control PCR should contain EasyXpress Positive-Control DNA (PCR) (white screw-cap), Positive-Control Sense Primer (white screw-cap), and Positive-Control Antisense Primer (white screw-cap). Products from this PCR should then be amplified using the No tag Sense Primer (yellow screw-cap) and 6xHis tag Antisense Primer (brown screw-cap). The final PCR product encodes the 32 kDa elongation factor EF-Ts with a C-terminal 6xHis tag.

Protocol: Two-Step PCR Procedure for Generating an Expression Template

This protocol is made up of two separate PCR procedures. In the first PCR, protein-specific sequence is used as a template. The primers used in this first PCR add sequences that will serve as hybridization sites in a second round of PCR (see Figure 7, page 19). In the second PCR, adapter primers (see Table 4) are used to add sequence that encodes affinity tags and/or regulatory elements required for efficient expression.

Important points before starting

- Wear gloves for all working steps in order to protect the reaction components from contaminating DNA and nucleases.
- Use DNase- and RNase-free filter pipet tips.
- Avoid using DNA templates that already contain promoter and terminator elements of the phage T7 gene 10. To avoid contamination of the second PCR with these elements, remove them from the protein-coding sequence using restriction enzymes. Separate the reaction products by agarose gel electrophoresis and purify the target sequence band from the agarose gel using the QIAGEN MinElute Gel Extraction Kit. Use the purified target sequence DNA for the first PCR.
- ProofStart DNA Polymerase requires an activation step of 5 min at 95°C.
- To amplify PCR products <2 kb, use values marked with a ●.
- To amplify PCR products >2 kb, use the values marked with a ▲.
- The optimal Mg^{2+} concentration should be determined empirically, but in most cases a concentration of 1.5 mM, as provided in the 1x ProofStart PCR Buffer, will produce satisfactory results.
- Q-Solution changes the melting behavior of DNA and can be used for PCR systems that do not work well under standard conditions. For detailed protocols see the *ProofStart PCR Handbook**.
- Lyophilized primers should be dissolved in a small volume of TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8.0) to make a concentrated stock solution.
- Prepare small aliquots of primer working solutions (10 µM) to avoid repeated thawing and freezing. Store all primer solutions at –20°C.

* All QIAGEN handbooks are available online in convenient and compact PDF format at www.qiagen.com/literature.

Procedure: First PCR using gene-specific primers

1. **Thaw 10x ProofStart PCR Buffer (blue screw-cap), dNTP mix (purple screw-cap), primer solutions, and, if required, 25 mM $MgSO_4$ (yellow screw-cap). Mix the solutions thoroughly before use.**
2. **Prepare a master mix according to Table 5.**
3. **Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes.**

   It is not necessary to keep PCR tubes on ice. ProofStart DNA Polymerase is inactive at room temperature.

Table 5. PCR Components (Master Mix and Template DNA)

Component	Volume/reaction	Final concentration
Master mix		
10x ProofStart PCR Buffer*	2.5 µl	1x
dNTP (10 mM of each)	0.75 µl	300 µM of each dNTP
Sense Primer (10 µM)	0.75–2.5 µl†	0.3–1 µM
Antisense Primer (10 µM)	0.75–2.5 µl†	0.3–1 µM
ProofStart DNA Polymerase	● 0.5 µl‡; ▲ 1 µl‡	● 1.25 units; ▲ 2.5 units
RNase-Free Water	variable	—
Template DNA		
Template DNA, added in step 4	variable	50–500 ng genomic DNA 1–100 ng cDNA§ 0.5–5.0 ng plasmid DNA
Total volume	**25 µl**	**—**

* contains 15 mM $MgSO_4$.

† The amount of primer used depends on the origin of the template DNA. Use 0.75 µl of a 10 µM solution of each primer when amplifying PCR products from plasmid DNA and 2.5 µl of a 10 µM solution of each primer when amplifying PCR products from genomic DNA or cDNA.

‡ Dependent on expected PCR product length. In general, use ● 0.5 µl enzyme when amplifying PCR products <2 kb and ▲ 1 µl enzyme when amplifying PCR products >2 kb.

§ The volume of the reverse transcription reaction added to the PCR should not exceed 10% of the total PCR volume.

4. **Add template DNA to the individual tubes containing the master mix.**

   For the positive control reaction use 1 µl of a 1 in 10 dilution of EasyXpress Positive control DNA (PCR) (white screw-cap) template. The resulting PCR fragment will have a length of 880 bp.

5. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 25 µl mineral oil.**

6. **Program the thermal cycler according to the manufacturer's instructions.**

   Each PCR program must start with an initial heat-activation step at 95°C for 5 min.

	Time	Temp.	Comments
Initial activation step	5 min	95°C	ProofStart DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation	1 min	94°C	
Annealing	1 min	50°C	Approximately 5°C below *Tm* of primers
Extension	● 1 min/kb	72°C	For PCR products of 1–2 kb, use an extension time of 1 min per kb DNA.
	▲ 2 min/kb	72°C	For PCR products >2 kb, use 2 min per kb DNA.
Number of cycles	30 or 40–45*		The number of cycles is dependent on the origin of the template DNA (see footnote)
Final extension:	7 min	72°C	
End of PCR cycling:	Indefinite	4°C	

* Use 30 cycles if amplifying PCR products from plasmid DNA and 40–45 cycles when amplifying PCR products from genomic DNA or cDNA.

7. **Place the PCR tubes in the thermal cycler and start the cycling program.**

   After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

8. **Analyze 1 µl PCR product on a 0.8–1.5% agarose gel.**

   The product of the first PCR should be the dominant band. Use 1 µl (approximately 100 ng DNA) of the first PCR product as template for the second PCR.

Procedure: Second PCR using adapter primers

1. **Thaw 10x ProofStart PCR Buffer (blue screw-cap), dNTP mix (purple screw-cap), primer solutions, and, if required, 25 mM $MgSO_4$ (yellow screw-cap). Mix the solutions thoroughly before use.**
2. **Prepare a master mix according to Table 6.**
3. **Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes.**

   It is not necessary to keep PCR tubes on ice. ProofStart DNA Polymerase is inactive at room temperature.

Table 6. PCR Components (Master Mix and Template DNA)

Component	Volume/reaction	Final concentration
Master mix		
10x ProofStart PCR Buffer*	2.5 µl	1x
dNTP (10 mM of each)	0.75 µl	300 µM of each dNTP
Sense Adapter Primer†	2 µl†	
Antisense Adapter Primer†	2 µl†	
ProofStart DNA Polymerase	● 0.5 µl‡; ▲ 1 µl‡	● 1.25 units; ▲ 2.5 units
RNase-Free Water	variable	—
Template DNA		
Template DNA, added in step 4 (~100 ng)	1–2 µl product from first PCR	
Total volume	**25 µl**	**—**

* Contains 15 mM $MgSO_4$

† For possible adapter primer combinations see Table 4, page 23.

‡ Dependent on expected PCR product length. In general, use ● 0.5 µl enzyme when amplifying PCR products <2 kb and ▲ 1 µl enzyme when amplifying PCR products >2 kb.

4. **Add template DNA (1–2 µl of the first PCR, ~100 ng) to the individual tubes containing the master mix.**
5. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 25 µl mineral oil.**

6. **Program the thermal cycler according to the manufacturer's instructions.**

   Each PCR program must start with an initial heat-activation step at 95°C for 5 min.

	Time	Temp.	Comments
Initial activation step	5 min	95°C	ProofStart DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation	1 min	94°C	
Annealing	1 min	50°C	Approximately 5°C below *Tm* of primers.
Extension	● 1 min/kb	72°C	For PCR products of 1–2 kb, use an extension time of 1 min per kb DNA.
	▲ 2 min/kb	72°C	For PCR products >2 kb, use 2 min per kb DNA.
Number of cycles	30		
Final extension:	7 min	72°C	
End of PCR cycling:	Indefinite	4°C	

7. **Place the PCR tubes in the thermal cycler and start the cycling program.**

   After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

8. **Analyze 1 µl of the first and second PCR on a 0.8–1.5% agarose gel.**

   The introduction of regulatory elements and affinity-tag sequences in the second PCR adds approximately 160–200 bp to the first PCR product.

9. **Determine the yield of the second PCR by comparing the product band to the molecular weight marker bands.**

   0.7 µg DNA (~7 µl of the second PCR) is required for a 50 µl in vitro translation reaction.

Protocol: In Vitro Translation Using a PCR Product as Template

This protocol is suitable for production of recombinant proteins using EasyXpress Protein Synthesis and Random Biotin Kits. We recommend that PCR products added to protein synthesis reactions are generated using the EasyXpress Linear Template Kit Plus. PCR products can be added directly to in vitro translation reactions without further cleanup.

Reactions can be easily scaled down to as little as 5 µl for high-throughput screening in 96-well microplate format. The reagent volumes given in Table 7 should be adjusted accordingly. We recommend using polypropylene 96-well plates (e.g., Greiner Bio-One cat. no. 650201) for reaction volumes >25 µl and 96-well thin-wall polycarbonate PCR plates (e.g., Corning Incorporated, cat. no. 6513) for reaction volumes <25 µl. To avoid evaporation, microplates and PCR plates should be sealed with a tape sheet during the in vitro translation reaction in an incubator or on a PCR cycler.

Materials and reagents to be supplied by user

- PCR template encoding the protein of interest and PCR template derived from control DNA (see protocol on page 24)
- Thermomixer (Eppendorf, Hamburg, Germany), water-bath, or heating block

Important general points before starting

- The in vitro translation system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- For multiple reactions, prepare a master mix without template, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the template.
- The *E. coli* extracts in EasyXpress Kits are very sensitive to multiple freeze–thaw cycles. The extracts are provided as individual aliquots in single tubes. Once thawed, keep on ice, and use within 4 hours. Do not thaw and refreeze more than two times.
- Except for the actual transcription-translation incubation, all handling steps should be carried out on ice.
- To determine the background level of protein synthesis, always include a no template control reaction (without template) in your experiment.

- The functionality of the kit can be checked by performing a positive-control reaction containing product from the second positive-control PCR (see protocol, page 24). This PCR product encodes the 32 kDa elongation factor EF-Ts with a C-terminal 6xHis tag. **Note**: expression levels of target proteins may be lower than those of the control protein.
- The recommended incubation temperature for protein synthesis is 37°C, but lower incubation temperatures may improve protein solubility in some cases.

Additional points to consider when producing biotinylated proteins

- Do not add Biotinyl-Lysyl-tRNA to a master mix. This may result in lower amounts of biotin incorporation due to deacylation of the tRNA carrying the biotinylated residue. Start the reaction by adding Biotinyl-Lysyl-tRNA to the master mix as recommended in the protocol.
- For multiple reactions, prepare a master mix without template and Biotinyl-Lysyl-tRNA, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the Biotinyl-Lysyl-tRNA and the template.
- Once thawed Biotinyl-Lysyl tRNA should be stored on ice and quickly returned to a –70°C freezer after use. Do not thaw and refreeze more than four times.

Procedure

1. **Thaw EasyXpress Reaction Buffer (blue screw-cap), *E coli* Extract (colorless snap-cap), and XE-Solution (green screw-cap) on ice. Thaw RNase-free water (colorless screw-cap) at room temperature (15–25°C). If using the EasyXpress Random Biotin Kit, thaw EasyXpress Biotinyl-Lysyl tRNA (Phe) (orange screw-cap) on ice.**

   Spin down and gently vortex all components before use. XE-Solution is a component of the EasyXpress Linear Template Kit Plus.

2. **Table 7 provides a pipetting scheme for EasyXpress protein synthesis reactions using PCR products. Calculate the amount of RNase-Free Water needed to bring the final reaction volume to 50 µl. Add the calculated amount of RNase-free water to each tube of *E. coli* extract, and mix by gently vortexing.**

Table 7. Pipetting Scheme for Setup of EasyXpress Protein Synthesis Reactions Using a PCR Product as a Template

Component	In vitro translation samples	Positive control	No-template control
E. coli Extract	17.5 µl	17.5 µl	17.5 µl
RNase-free water	Add to 50 µl	Add to 50 µl	Add to 50 µl
XE-Solution*	2 µl	2 µl	2 µl
DNA template from second PCR	0.7 µg	7 µl	–
EasyXpress Reaction Buffer	20 µl	20 µl	20 µl
When producing randomly biotinylated proteins: Biotinyl-Lysyl-tRNA (Phe)	3 µl	3 µl	3 µl
Total	**50 µl**	**50 µl**	**50 µl**

* Mix XE-Solution with the second PCR product before adding to the reaction. It is important that XE-Solution is not added directly to the diluted *E. coli* extract.

3. **For each reaction add 2 µl XE-Solution to 0.7 µg DNA (~7 µl) from the second PCR in a separate tube and mix by pipetting up and down. For the positive-control reaction add 2 µl XE-Solution to 7 µl from the positive-control second PCR and mix by pipetting up and down. Do not add any DNA to the no-template control reaction.**

   It is important that XE-Solution is not added directly to the diluted *E. coli* Extract. For the no-template control reaction, mix 2 µl XE-Solution and 7 µl RNase-free water.

4. **Add the XE-Solution-PCR product mix from step 3 to each reaction, and mix by gently vortexing.**
5. **Add 20 µl of Reaction Buffer to the mix from step 4.**
6. **If producing biotinylated proteins using the EasyXpress Random Biotin Kit, add 3 µl EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each reaction. Otherwise proceed to step 7.**

   It is important that the EasyXpress Biotinyl-Lysyl tRNA (sense) is the last reaction component added.

7. **Mix and centrifuge briefly to collect reactions in the bottom of the tubes.**
8. **Incubate the reactions at 37°C for 1 h.**

   To achieve optimal distribution of reaction components, samples should be shaken in a thermomixer or water-bath shaker.
9. **Proceed with sample analysis.**

   Biotin labeled proteins can be detected after separation on SDS-PAGE and subsequent blotting by colorimetric or chemiluminescent visualization with Streptavidin-AP or Streptavidin-HRP. Usually 1–5 µl of crude lysate is sufficient for one gel lane.

Protocol: In Vitro Translation Using Plasmid DNA as Template

This protocol is suitable for production of recombinant proteins using EasyXpress Protein Synthesis and Random Biotin Kits.

Reactions can be easily scaled down to as little as 5 µl for high-throughput screening in 96-well microplate format. The reagent volumes given in Table 8 should be adjusted accordingly. We recommend using polypropylene 96-well plates (e.g., Greiner Bio-One, cat. no. 650201) for reaction volumes >25 µl and 96-well thin-wall polycarbonate PCR plates (e.g., Corning Incorporated, cat. no. 6513) for reaction volumes <25 µl. To avoid evaporation, microplates and PCR plates should be sealed with a tape sheet during the in vitro translation reaction in an incubator or on a PCR cycler.

Materials and reagents to be supplied by user

- Plasmid DNA template encoding the protein of interest
- Thermomixer (Eppendorf, Hamburg, Germany), water-bath, or heating block

Important general points before starting

- The in vitro translation system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- For multiple reactions, prepare a master mix without template, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the template.
- The *E. coli* extracts in EasyXpress Kits are very sensitive to multiple freeze–thaw cycles. The extracts are provided as individual aliquots in single tubes. Once thawed, keep on ice, and use within 4 hours. Do not thaw and refreeze more than two times.
- Except for the actual transcription-translation incubation, all handling steps should be carried out on ice.
- To determine the background level of protein synthesis, always include a no template control reaction (without template) in your experiment.

- The functionality of the kit can be checked by performing a positive-control reaction using EasyXpress Positive Control DNA. This plasmid encodes the 32 kDa elongation factor EF-Ts with a C-terminal 6xHis tag. **Note**: expression levels of target proteins may be lower than those of the control protein.
- The recommended incubation temperature for protein synthesis is 37°C, but lower incubation temperatures may improve protein solubility in some cases.

Additional points to consider when producing biotinylated proteins

- Do not add Biotinyl-Lysyl-tRNA to a master mix. This may result in lower amounts of biotin incorporation due to deacylation of the tRNA carrying the biotinylated residue. Start the reaction by adding Biotinyl-Lysyl-tRNA to the master mix as recommended in the protocol.
- For multiple reactions, prepare a master mix without template and Biotinyl-Lysyl-tRNA, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the Biotinyl-Lysyl-tRNA and the template.
- Once thawed Biotinyl-Lysyl tRNA should be stored on ice and quickly returned to a –70°C freezer after use. Do not thaw and refreeze more than four times.

Procedure

1. **Thaw EasyXpress Reaction Buffer (blue screw-cap) and *E. coli* Extract (colorless snap-cap) on ice. Thaw RNase-Free Water (colorless screw-cap) at room temperature (15–25°C). If using the EasyXpress Random Biotin Kit, thaw EasyXpress Biotinyl-Lysyl tRNA (Phe) (orange screw-cap) on ice.**

   Spin down and gently vortex all components before use.

2. **Table 8 provides a pipetting scheme for EasyXpress protein synthesis using plasmid DNA. Calculate the amount of RNase-Free Water needed to bring the final reaction volume to 50 µl. Add the calculated amount of RNase-Free Water to each tube of *E. coli* Extract, and mix by gently vortexing.**

Table 8. Pipetting Scheme for Setup of EasyXpress Protein Synthesis Reactions Using Plasmid DNA as a Template

Component	In vitro translation samples	Positive control*	No-template control
E. coli Extract	17.5 µl	17.5 µl	17.5 µl
RNase-free water	Add to 50 µl	Add to 50 µl	Add to 50 µl
DNA template	5–10 nM	2.5 µl	–
EasyXpress Reaction Buffer	20 µl	20 µl	20 µl
When producing randomly biotinylated proteins: Biotinyl-Lysyl-tRNA (Phe)	3 µl	3 µl	3 µl
Total	**50 µl**	**50 µl**	**50 µl**

* EasyXpress Positive-Control DNA (yellow screw-cap) supplied with the kit.

3. **Add 5–10 nM (~0.5–1 µg) plasmid DNA to each reaction, and mix by gently vortexing.**
4. **Add 20 µl of Reaction Buffer to the mix from step 3, and mix by gently vortexing.**
5. **If producing biotinylated proteins using the EasyXpress Random Biotin Kit, add 3 µl EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each reaction. Otherwise proceed to step 6.**

   It is important that the EasyXpress Biotinyl-Lysyl tRNA (sense) is the last reaction component added.
6. **Mix and centrifuge briefly to collect reactions in the bottom of the tubes.**
7. **Incubate the reactions at 37°C for 1 h.**

   To achieve optimal distribution of reaction components, samples should be shaken in a thermomixer or water-bath shaker.
8. **Proceed with sample analysis.**

   Biotin labeled proteins can be detected after separation on SDS-PAGE and subsequent blotting by colorimetric or chemiluminescent visualization with Streptavidin-AP or Streptavidin-HRP. Usually 1–5 µl of crude lysate is sufficient for one gel lane.

Protocol: Scaling Up In Vitro Translation Reactions Using the EasyXpress Protein Synthesis Maxi Kit

This protocol is suitable for the in vitro production of recombinant proteins from plasmid DNA using the EasyXpress Protein Synthesis Maxi Kit in a 1 ml volume. If a smaller or larger reaction volume is desired the volumes of the components should be scaled up and down accordingly. Smaller volume in vitro translation reactions (50–500 µl) should be performed in 1.5 ml reaction tubes. Translation reactions >500 µl should be incubated in 14 ml reaction tubes.

Materials to be supplied by user

- Plasmid DNA template encoding the protein of interest
- Thermomixer, incubator, or water-bath
- Sterile 1.5 ml or 14 ml reaction tubes

Important points before starting

- The in vitro translation system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- For multiple reactions, prepare a master mix without template, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the template.
- The *E. coli* extract in the EasyXpress Protein Synthesis Kit is sensitive to multiple freeze–thaw cycles. The extracts are provided as individual aliquots in single tubes. Once thawed, use *E. coli* extract within 4 hours. Do not refreeze and thaw more than four times.
- Except for the actual transcription-translation incubation, all handling steps should be carried out on ice.
- To determine the background level of protein synthesis, always include a no-template control reaction in your experiment.
- The functionality of the kit can be checked by performing a positive-control reaction containing EasyXpress Positive-Control DNA that encodes the 32 kDa elongation factor EF-Ts with a C-terminal 6xHis tag.
- The recommended incubation temperature for protein synthesis is 37°C, but lower incubation temperatures may improve protein solubility in some cases.

Procedure

1. **Thaw EasyXpress *E. coli* Extract and Reaction Buffer (blue screw-cap) on ice. Thaw RNase-free water (colorless screw-cap) and EasyXpress Positive–Control DNA (yellow screw-cap) at room temperature (15–25°C).**

   Spin down and gently vortex all components before use.

2. **Table 9 provides a pipetting scheme for EasyXpress reactions using plasmid DNA. Calculate the amount of RNase-free water needed to bring the final reaction volume to 1 ml. Add the calculated amount of RNase-free water to a 14 ml tube containing *E. coli* Extract (350 µl for a 1 ml reaction), and mix by gently vortexing.**

Table 9. Pipetting Scheme for Setup of EasyXpress Protein Synthesis Reactions Using the EasyXpress Protein Synthesis Maxi Kit

Component	In vitro translation samples	Positive control*	No-template control
E. coli Extract	350 µl	350 µl	350 µl
RNase-free water	Add to 1 ml	200 µl	250 µl
DNA template	5–10 nM	50 µl	–
EasyXpress Reaction Buffer	400 µl	400 µl	400 µl
Total	**1000 µl**	**1000 µl**	**1000 µl**

* EasyXpress Positive-Control DNA (yellow screw-cap) supplied with the kit

3. **Add 5–10 nM (~10–20 µg) plasmid DNA to each reaction, and mix by gently vortexing.**
4. **Add 400 µl of Reaction Buffer to the mix from step 3, and mix by gently vortexing.**
5. **Incubate the reactions at 37°C for 1 h.**

   To achieve optimal distribution of reaction components, samples should be shaken in a thermomixer or water-bath shaker.

6. **Proceed with sample analysis.**

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocol(s) in this handbook or molecular biology applications (see back cover for contact information). Many of the PCR-related problems in this guide are comprehensively covered in the *ProofStart PCR Handbook*, which can be viewed and downloaded in convenient PDF format from the QIAGEN website.

		Comments and suggestions
First and second PCR		
Little or no product		
a)	ProofStart DNA Polymerase not activated at 95°C for 5 min	Check if PCR was started with an initial 95°C incubation step.
b)	Pipetting error or missing reagent	Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers and dNTP mix.
c)	Insufficient starting template	Increase amount of starting template used in PCR.
d)	PCR conditions not optimal	Using the same cycling conditions, repeat the PCR using Q-Solution. Follow the protocol in the *ProofStart PCR Handbook*.
e)	Primer concentration not optimal	For calculation of primer concentration, refer to appendix in the *ProofStart PCR Handbook*.
f)	Extension time too short	Increase extension time in increments of 1 min. Ensure that an extension time of 2 min per kb DNA was used for fragments >2 kb.
g)	Enzyme concentration too low	For PCR fragments <2 kb, use 0.5 µl ProofStart DNA Polymerase per 25 µl reaction. For PCR fragments >2 kb, use 1 µl of enzyme per 25 µl reaction.
h)	Insufficient number of cycles	Increase the number of cycles in steps of 5 cycles.
i)	Problems with starting template	Check the concentration, storage conditions, and quality of starting template. If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat PCR using the new stock solutions.

		Comments and suggestions
j)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM $MgSO_4$.
k)	Primer design not optimal	Review primer design, see page 21.
l)	Incorrect dNTP concentration	Ensure that a dNTP concentration of 300 µM of each dNTP was used. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.
Product is multi-banded		
a)	PCR cycling conditions not optimal	Review primer design, see page 21.
b)	Enzyme concentration too low	For PCR fragments <2 kb, use 0.5 µl ProofStart DNA Polymerase per 25 µl reaction. For PCR fragments >2 kb, use 1 µl of enzyme per 25 µl reaction.
c)	Extension time too short	Increase extension time in increments of 1 min. Ensure that an extension time of 2 min per kb DNA was used for fragments >2 kb.
d)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM using the 25 mM $MgSO_4$ solution provided (see *ProofStart PCR Handbook*).
e)	Primer concentration not optimal or primers degraded	For calculation of primer concentration, refer to the appendix, in the *ProofStart PCR Handbook*. Particularly when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.
f)	Primer design not optimal	Review primer design, see page 21.
g)	Incorrect dNTP concentration	Ensure that a dNTP concentration of 300 µM of each dNTP was used. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.

		Comments and suggestions
Product is smeared		
a)	Insufficient starting template	Increase amount of starting template used in PCR.
b)	Extension time too short	Increase extension time in increments of 1 min. Ensure that an extension time of 2 min per kb DNA was used for fragments >2 kb.
c)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM using the 25 mM $MgSO_4$ solution provided (see *ProofStart PCR Handbook*).
d)	dNTP concentration not optimal	Ensure that a dNTP concentration of 300 µM of each dNTP was used. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.
e)	Primer design not optimal	Review primer design, see page 21.
In vitro translation		
No control protein visible		
a)	Reaction was contaminated with RNases	Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
b)	Kit has not been stored at –70°C	The *E. coli* extracts in EasyXpress Kits will remain active for just 24 h if stored at temperatures of –20°C or above. After this time the extracts will lose activity.
c)	Pipetting error or missing reagent	Check the pipets used for experimental setup. Mix all reagents well after thawing, and repeat in vitro translation.
d)	Incorrect setup temperature	Be sure to set up the reaction on ice.

No target protein but normal expression of control protein

a)	Poor quality or wrong quantity of DNA template	Check the concentration, integrity, and purity of the DNA template. Prepare high-purity plasmid DNA with QIAGEN plasmid kits and optimized linear expression templates with the QIAGEN EasyXpress Linear Template Kit Plus. Determine the optimal amount of DNA template used in the in vitro translation by titration.
b)	DNA template not optimally configured, or error in cloning	Check the sequence. Make sure that the start codon is in the right position for expression. For linear expression templates generated using the EasyXpress Linear Template Kit Plus check the strategy for designing gene-specific primers.
c)	In vitro transcription or in vitro translation is disrupted by expressed protein	Express control protein in the presence of the target protein. If expression of control protein is inhibited, it may not be possible to express the target protein using the EasyXpress Protein Synthesis System.
d)	Rigid secondary structures in the mRNA inhibit initiation of translation	Include a 6xHis-tag coding sequence at the 5' end of the protein coding sequence. If the protein to be expressed already contains a tag, move the tag to the opposite terminus.

Low expression yield*

a)	Contamination with RNases	Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
b)	Poor quality or wrong quantity of DNA template	Check the concentration, integrity, and purity of the DNA template. Prepare high-purity plasmid DNA with QIAGEN plasmid kits and optimized linear expression templates with the QIAGEN EasyXpress Linear Template Kit Plus. Determine the optimal amount of DNA template used in the in vitro translation by titration.
c)	If protein is expressed with a tag, the tag has a negative effect on expression of the protein	Introduce different tag sequences at different positions (N-terminal or C-terminal) via PCR using the EasyXpress Linear Template Kit Plus. This can significantly affect protein expression and solubility (2).

* Expression levels of target proteins may be lower than those of the control protein.

		Comments and suggestions
d)	Low initiation of translation due to rigid secondary structures in the mRNA	Include a 6xHis-tag coding sequence at the 5' end of the protein coding sequence. If the protein to be expressed already contains a tag, move the tag to the opposite terminus.
e)	Plasmid DNA template contains *E. coli* promoter (e.g., T5 or lac)	Add IPTG (final concentration 1 mM) to translation reaction to overcome endogenous lac repressor. Increase the template concentration to 10 nM final concentration.
Sufficient protein expression, but low yield of active protein		
a)	Incorrect folding of the protein due to dependence on posttranslational modifications	*E. coli* lysate cannot introduce posttranslational modifications like glycosylation, phosphorylation, or signal-peptide cleavage.
b)	Cofactors required for activity	Add cofactors to synthesis reaction and/or activity assay.
Expressed protein is insoluble		
Protein forms aggregates		We recommended a 37°C incubation temperature for protein synthesis, but lower incubation temperatures may improve protein solubility.
Expressed protein		
a)	Premature termination of translation	Check reading frame of the target sequence for mutations that might produce a stop codon.
b)	Protein is degraded by proteases	Add protease inhibitors to the in vitro translation reaction.
No biotin incorporation		
No codon for biotin incorporation present		Ensure that the template contains at least one Phe (UUC) codon within the coding sequence of the protein. Check for biotin incorporation by performing a control reaction using the control plasmid and BioLys-tRNA followed by western blotting. Develop the western blot using an appropriate method (e.g., streptavidin-HRP). Perform positive controls to ensure that all compounds used for detection are functioning correctly.

Poor incorporation of biotin/weak signal on western blot

a)	The incorporated biotin residue is not accessible to detection reagents	Consider using the Easy Express Site-Specific Biotin Kit which enables a free choice of biotin position.
b)	BioLys-tRNA added to master mix	Do not add BioLys-tRNA to a master mix. This may result in lower amounts of biotin incorporation due to deacylation of the tRNA carrying the biotinylated residue. Start the reaction by adding BioLys-tRNA to the master mix as recommended in the protocol.

Appendix A: Purification of In Vitro Synthesized Proteins

Purification of 6xHis-tagged Proteins Using Ni-NTA Magnetic Agarose Beads

This protocol can be used to efficiently purify 6xHis-tagged proteins from 50 µl EasyXpress in vitro translation reactions.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- In vitro translation reaction containing 6xHis-tagged protein
- Ni-NTA Magnetic Agarose Beads (QIAGEN cat. no. 36111)
- Magnetic separator (e.g., 12 tube-magnet, QIAGEN cat. no. 36912)
- Buffer NPI-10-T (Ni-NTA Beads Binding Buffer)
- Buffer NPI-20-T (Ni-NTA Beads Wash Buffer)
- Buffer NPI-250-T (Ni-NTA Beads Elution Buffer)

Buffer compositions are provided in the Appendix on page 58.

Procedure

1. **Resuspend Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately pipet 150 µl of the 5% Ni-NTA Magnetic Agarose Bead suspension into a 1.5 ml reaction tube.**

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly. 100 µl magnetic bead suspension has a binding capacity of 30 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly. However, use of volumes less than 10 µl are not recommended due to the associated handling problems — smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

2. **Place the reaction tube on a magnetic separator for 1 min. Carefully remove supernatant with a pipet.**
3. **Remove the tube from the magnetic separator and add 500 µl Buffer NPI-10-T. Briefly vortex, place the reaction tube on a magnetic separator for 1 min, and remove supernatant.**

4. **Pipet 700 µl Buffer NPI-10-T into the tube containing the Ni-NTA Magnetic Agarose Beads and mix by pipetting up and down.**
5. **Pipet the 50 µl in vitro translation reaction into the tube containing the Ni-NTA Magnetic Agarose Beads suspension.**
6. **Mix the suspension gently on an end-over-end shaker for 60 min at 4°C.**
7. **Place the tube on a magnetic separator for 1 min and remove supernatant with a pipet.**

   Tubes may be briefly centrifuged before placing on the magnetic separator, to collect droplets of suspension from the tube caps.
8. **Remove tube from the magnet, add 500 µl of Buffer NPI-20-T, mix the suspension, place the tube on a magnetic separator for 1 min, and remove buffer.**
9. **Repeat step 8 one or two times.**

   Buffer remaining after the final wash should be removed completely.
10. **Add 50 µl of Buffer NPI-250-T to the beads, mix the suspension, incubate the tube for 1 min, place for 1 min on magnetic separator, and collect the eluate.**

    Tubes may be centrifuged before placing on the magnetic separator, to collect droplets of suspension from the tube caps.
11. **Repeat step 10.**

    Most of the 6xHis-tagged protein will elute in the first elution step.

Purification of 6xHis-tagged Proteins Using Ni-NTA Magnetic Agarose Beads in 96-Well Plates

This protocol can be used to efficiently purify 6xHis-tagged proteins from 25 µl EasyXpress in vitro translation reaction aliquots in 96-well microplates.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- In vitro translation reactions containing 6xHis-tagged protein
- Ni-NTA Magnetic Agarose Beads (QIAGEN cat. no. 36111)
- Flat-bottom microplates (e.g., 96-Well Microplate FB, QIAGEN cat. no. 36985)
- Magnetic separator (e.g., 96-Well Magnet Type A, QIAGEN cat. no. 36915)
- Buffer NPI-10-T (Ni-NTA Beads Binding Buffer)
- Buffer NPI-20-T (Ni-NTA Beads Wash Buffer)
- Buffer NPI-250-T (Ni-NTA Beads Elution Buffer)

Buffer compositions are provided in the Appendix on page 58.

Procedure

1. **Resuspend Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately pipet 75 µl of 5% Ni-NTA Magnetic Agarose Bead suspension into each well of a flat-bottom 96-well microplate.**

   We recommend use of 96-well microplates that have flat-bottom wells. Flat-bottom wells are large enough to contain the recommended volumes and provide optimal mixing efficiency.

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly. 100 µl magnetic-bead suspension has a binding capacity of 30 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly. However, use of volumes less than 10 µl are not recommended due to the associated handling problems — smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

2. **Place the 96-well microplate on the magnetic separator for 1 min. Carefully remove supernatant with a pipet.**
3. **Remove the 96-well microplate from the magnet and pipet 200 µl Buffer NPI-10-T into each well. Mix on microplate shaker for 2 s, place the 96-well microplate on a magnet for 1 min, and remove supernatant.**
4. **Pipet a 25 µl in vitro translation reaction aliquot into each well containing Ni-NTA Magnetic Beads.**
5. **Mix the suspension gently on microplate shaker for 60 min at 4°C.**
6. **Place the 96-well microplate on the magnet for 1 min. Carefully remove supernatant with a pipet.**
7. **Pipet 200 µl of Buffer NPI-20-T into each well, mix on a microplate shaker for 2 s, place on the magnet for 1 min, and remove buffer.**
8. **Repeat step 7.**
9. **Add 50 µl of Buffer NPI-250-T to each well, mix on a microplate shaker for 2 s, incubate for 1 min, place on the magnet for 1 min, and collect the eluate.**
10. **Repeat step 9.**

    Most of the 6xHis-tagged protein will elute in the first elution step.

Purification of 6xHis-tagged Proteins Using Ni-NTA Superflow Under Native Conditions

This protocol can be used to efficiently purify 6xHis-tagged proteins from 1ml EasyXpress in vitro translation reactions.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- In vitro translation reaction containing 6xHis-tagged protein
- Ni-NTA Superflow (QIAGEN cat. no. 30410)
- Buffer NPI-10 (Ni-NTA Superflow Binding Buffer)
- Buffer NPI-20 (Ni-NTA Superflow Wash Buffer)
- Buffer NPI-250 (Ni-NTA Superflow Elution Buffer)
- Empty column
- 14 ml reaction tube

Buffer compositions are provided in the Appendix on page 58.

Procedure

1. **Pipet 2 ml Buffer NPI-10 into a 14 ml tube.**
2. **Pipet 500 µl of 50% Ni-NTA Superflow slurry into the tube containing binding buffer. Pipet 1 ml in vitro translation reaction into the Superflow suspension and mix gently by shaking (200 rpm on a rotary shaker) at 4°C for 60 min.**

   Note: Generally the binding capacity of Ni-NTA Superflow is 5–10 mg protein per ml resin.
3. **Load the reaction–Ni-NTA mixture into a column with the bottom outlet capped.**
4. **Remove bottom cap and collect the column flow-through.**

   Save flow through for SDS-PAGE analysis, if desired.
5. **Wash column bed twice with 2 ml Buffer NPI-20.**

   Collect wash fractions for SDS-PAGE analysis, if desired.
6. **Elute the protein 4 times with 0.25 ml Buffer NPI-250. Collect the eluates in four tubes and analyze by SDS-PAGE.**

Purification of *Strep*-tagged Proteins Using *Strep*-Tactin Magnetic Beads

This protocol can be used to efficiently purify *Strep*-tagged proteins from 50 µl EasyXpress in vitro translation reactions.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- In vitro translation reactions containing *Strep*-tagged protein
- *Strep*-Tactin Magnetic Beads (e.g., QIAGEN cat. no. 36311)
- Magnetic separator (e.g., 12 tube-magnet, QIAGEN cat. no. 36912)
- Buffer NP-T (*Strep*-Tactin Beads Wash Buffer)
- Buffer NPB-T (*Strep*-Tactin Beads Elution Buffer)

Buffer compositions are provided in the Appendix on page 58.

Procedure

1. **Resuspend *Strep*-Tactin Magnetic Beads by vortexing for 2 s and then immediately pipet 200 µl of 10% *Strep*-Tactin Magnetic Bead suspension into a 1.5 ml reaction tube.**
2. **Place the reaction tube on a magnetic separator for 1 min. Remove supernatant carefully with a pipet.**
3. **Remove the tube from the magnetic separator and pipet 500 µl Buffer NP-T into the tube containing the beads. Briefly vortex, place the reaction tube on a magnetic separator for 1 min, and remove supernatant.**
4. **Add 50 µl in vitro translation reaction to the *Strep*-Tactin Magnetic Beads.**
5. **Mix the suspension gently shaking (200 rpm on a rotary shaker) for 60 min at 4°C.**

   After mixing, tubes may be centrifuged briefly to collect droplets of suspension from the tube caps.
6. **Place the tube on a magnetic separator for 1 min and remove supernatant with a pipet.**
7. **Remove tube from the magnet, pipet 500 µl Buffer NP-T into the tube, gently vortex the suspension, place the tube on a magnetic separator for 1 min, and remove buffer.**

8. **Repeat step 7.**
9. **Pipet 50 µl Buffer NPB-T into the tube containing the beads, gently vortex the suspension, incubate the tube for 5 min, place the tube on a magnetic separator for 1 min, and collect the eluate in a clean tube.**
10. **Repeat step 9 to give two elution fractions.**

    Because the biotin used for elution binds *Strep*-Tactin with extremely high affinity, it is not possible to regenerate *Strep*-Tactin Magnetic Beads.

Purification of *Strep*-tagged Proteins Using *Strep*-Tactin Superflow

This protocol can be used to efficiently purify *Strep*-tagged proteins from 1 ml EasyXpress in vitro translation reactions. This protocol can also be used to further purify proteins carrying both a 6xHis-tag and *Strep*-tag (His·*Strep*-tagged proteins) that have been eluted from Ni-NTA Superflow.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Pooled Ni-NTA Superflow eluates/in vitro translation reaction containing *Strep*-tagged proteins
- Empty 5 ml polypropylene column
- *Strep*-Tactin Superflow (e.g., QIAGEN, cat. no. 30001)
- Buffer NP (*Strep*-Tactin Superflow Wash Buffer)
- Buffer NPD (*Strep*-Tactin Superflow Elution Buffer)
- 14 ml reaction tube

Buffer compositions are provided in the Appendix on page 58.

Procedure

1. **Pipet eluates or 1 ml in vitro translation reaction into a 14 ml tube and add 1 ml *Strep*-Tactin Superflow Resin suspension. Mix gently by shaking (200 rpm on a rotary shaker) at 4°C for 60 min.**
2. **Load the *Strep*-Tactin Superflow resin into a column with the bottom outlet capped.**
3. **Remove bottom cap and collect the flow-through.**

   Save flow-through for SDS-PAGE analysis.

4. **Wash column two times with 2 ml Buffer NP.**

   Collect wash fractions for SDS-PAGE analysis.

5. **Elute the protein six times with 0.25 ml Buffer NPD. Collect eluates in six tubes and analyze by SDS-PAGE.**

   Strep-Tactin Superflow can be regenerated using the procedure provided in the Appendix on page 60.

Appendix B: Adapter Primer Sequences and Multiple Cloning Sites

No tag Sense Primer

5'–ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACggTTTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA–'3

H_2N — Protein

***Strep*-tag Sense Primer**

5'–ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACggTTTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA**ATg**TggTCTCATCCgCAATTCgAAAAAAgCgCTgAAAACCTgATCgAAggCCgT'3

H_2N — MWSHPQFEKSAENLIEGR(↓) — Protein

6xHis tag Sense Primer

5'ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACggTTTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA**ATg**AAACATCATCACCATCACCACTCgACCCACgCgCATgTCgTAAAAAgCACCCAA–'3

H_2N—MKHHHHHHSTHAHAHVVKSTQ — Protein

No tag Antisense Primer

5'–**TAA**TAACTAACTAACCAAgATCTgTACCCCTTggggCCTCTAAACgggTCTTgAggggTTTTTTggATCCgAATTCACCggTgATATCAT–3'

Protein — Stop — COOH

Strep-tag Antisense Primer

5'–TCTgCCTggTCTCATCCgCAATTCgAAAAA**TAA**TAACTAACTAACCAAgATCTgTACCCCTT
ggggCCTCTAAACgggTCTTgAggggTTTTTTggATCCgAATTCACCggTgATATCAT–3'

Protein — SAWSHPQFEK — Stop — COOH

6xHis tag Antisense Primer

5'–TggggTCACCACCATCACCATCAT**TAA**TAACTAACTAACCAAgATCTgTACCCCTTgggg
CCTCTAAACgggTCTTgAggggTTTTTTggATCCgAATTCACCggTgATATCAT–3'

Protein — WGHHHHHH — Stop — COOH

Underlined sequence hybridizes to 5' tails of gene-specific primers.

Sequencing primers

M13 forward (–20)	GTAAAACGACGGCCAGT
M13 reverse (–21)	CAGGAAACAGCTATGAC

Cloning PCR Products Generated with the EasyXpress Linear Template Kit Plus into the EasyXpress pIX3.0 Vector

The EasyXpress pIX3.0 vector is designed for cloning of PCR products generated by the EasyXpress Linear Template Kit plus. The PCR products contain multiple cloning sites that are compatible with the multiple cloning site of the pIX3.0 vector.

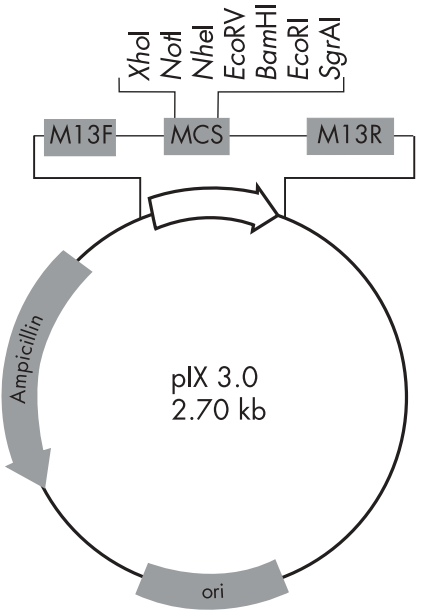

Figure 8 The pIX3.0 Vector.

EasyXpress Sense Primer Multiple Cloning Site

*Eco*RV *Xho*I *Not*I *Nhe*I

5'–ATGATATCTCGAGCGGCCGCTAGCT–3'
3'–TACTATAGAGCTCGCCGGCGATCGA–5'

EasyXpress Antisense Primer Multiple Cloning Site

*Bam*HI *Eco*RI *Sgr*AI *Eco*RV

5'–TGGATCCGAATTCTCCGGTGATATCAT–3'
3'–ACCTAGGCTTAAGTGGCCACTATAGTA–5'

EasyXpress pIX3.0 Vector Multiple Cloning Site

*Xho*I *Not*I *Nhe*I *Eco*RV *Bam*HI *Eco*RI *Sgr*AI

5'–CTCGAGCGGCCGCTAGCATGCCATGGATATCAGGCCTGGATCCGAATTCACCGGTG–3'
3'–GAGCTCGCCGGCGATCGTACGGTACCTATAGTCCGGACCTAGGCTTAAGTGGCCAC–5'

Appendix C: Optimization of EasyXpress Small-Scale Reactions

Although the EasyXpress system has been developed to give the highest yields of active and soluble protein, it may be possible to further optimize the synthesis procedure for individual proteins, (i.e., the total expression or the solubility may be increased by including additives in the synthesis reaction).

As the response to additives is protein-dependent, no general recommendation can be provided. Conditions that give improved results in small-scale reactions should then be transferred linearly to the large-scale reaction (e.g., final additive concentrations showing a positive effect in small-scale reactions should be maintained in the large-scale reaction). The table below gives some examples of reagents and reaction conditions that may lead to improved results with regard to protein solubility and/or yield.

Reaction condition	Optimization suggestions
Template concentration	Titrate between 0.2 and 2 µg per 50 µl reaction
Presence of IPTG	Usually not required, can be added to 1 mM
Temperature	Reaction can be performed at temperatures from 15–37°C
Presence of detergents*	Detergents can be added to a final concentration of 0.05–0.1% (v/v)
Presence of cofactors	Add as required (e.g., $ZnCl_2$ can be added to a final concentration of 0.1–0.5 mM)
Presence of glycerol	Can be added to a final concentration of 3% (v/v)

* Some detergents may reduce efficiency of protein expression.

Appendix D: Incorporating Radioactive Labels into Proteins for Quantification

Protein expressed using the EasyXpress Protein Synthesis Kit can be quantified by incorporating radioactive amino acids. It is recommended that 0.15 µCi ^{14}C-labeled leucine (^{14}C-Leu) (300 mCi/mmol) is added per 50 µl reaction. A protocol and example calculation of protein yield is given below. This protocol can be used for reactions where plasmid DNA or a PCR product is used as a template.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Plasmid DNA or PCR product encoding protein of interest
- Thermomixer, water-bath, or heating block
- ^{14}C-labeled leucine (^{14}C-Leu, 300 mCi/mmol)

Procedure

1. Thaw EasyXpress *E. coli* Extract and Reaction Buffer (blue screw-cap) on ice. If using a PCR product as a template, thaw XE-Solution (green screw-cap) on ice. Thaw RNase-free water (colorless screw-cap) and EasyXpress Positive-Control DNA (yellow screw-cap) at room temperature (15–25°C).

2. Tables 10 and 11 provide pipetting schemes for EasyXpress radioactive labeling reactions using plasmid DNA or PCR products. Calculate the amount of RNase-free water needed to bring the final reaction volume to 50 µl. Add the calculated amount of RNase-free water to each tube of *E. coli* extract, and mix by gently vortexing.

3. If using plasmid DNA as a template, proceed using step 3a. If using a PCR product as a template, proceed using step 3b.

3a. Add 5–10 nM (0.5–1.0 µg) plasmid DNA template to each reaction tube. Add 2.5 µl EasyXpress Positive-Control DNA to the positive-control reaction tube. Do not add any DNA to the no-template control reaction tube. Mix reactions by gently vortexing.

3b. For each reaction add 2 µl XE-Solution to 0.7 µg DNA (~7 µl) from the second PCR in a separate tube and mix by pipetting up and down. For the positive-control reaction add 2 µl XE-Solution to 7 µl from the positive-control second PCR and mix by pipetting up and down. Do not add any DNA to the no-template control reaction. Mix reactions by gently vortexing.

It is important that XE-Solution is not added directly to the diluted *E. coli* extract. For the no-template control reaction, mix 2 µl XE-Solution and 7 µl RNase-free water.

Table 10. Pipetting Scheme for Setup of EasyXpress Protein Synthesis Reactions Using a Radioactive Label and a PCR Product as Template

Component	In vitro translation samples	Positive control	No-template control
E. coli Extract	17.5 µl	17.5 µl	17.5 µl
RNase-free water	Add to 50 µl	0.5 µl	7.5 µl
XE-Solution*	2 µl	2 µl	2 µl
DNA template from second PCR	0.7 µg	7 µl	–
EasyXpress Reaction Buffer	20 µl	20 µl	20 µl
^{14}C-Leu (300mMCi/mmol)	3 µl (=0.15 µCi)	3 µl (=0.15 µCi)	3 µl (=0.15 µCi)
Total	**50 µl**	**50 µl**	**50 µl**

* Mix XE-Solution with the second PCR product before adding to the reaction. It is important that XE-Solution is not added directly to the diluted *E. coli* extract.

4. **Add 20 µl of Reaction Buffer to the reactions from step 3a or 3b, and mix by gently vortexing.**
5. **Add 3.0 µl (= 0.15 µCi) ^{14}C-Leu (300 mCi/mmol) to the reactions from step 4.**
6. **Mix and centrifuge briefly to collect reactions in the bottom of the tubes.**
7. **Incubate the reactions at 37°C for 1 h.**

   To achieve optimal distribution of reaction components, samples should be shaken in a thermomixer or water-bath shaker.
8. **Proceed with sample analysis.**

Table 11. Pipetting Scheme for Setup of EasyXpress Protein Synthesis Reactions Using a Radioactive Label and a Plasmid DNA Template

Component	In vitro translation samples	Positive control*	No-template control
E. coli Extract	17.5 µl	17.5 µl	17.5 µl
RNase-free water	Add to 50 µl	7 µl	9.5 µl
Plasmid DNA template	5–10 nM	2.5 µl	–
EasyXpress Reaction Buffer	20 µl	20 µl	20 µl
^{14}C-Leu (300mMCi/mmol)	3 µl (=0.15 µCi)	3 µl (=0.15 µCi)	3 µl (=0.15 µCi)
Total	**50 µl**	**50 µl**	**50 µl**

* EasyXpress Positive-Control DNA (yellow-screw cap) supplied with the kit.

Determination of Protein Yield by TCA Precipitation and Scintillation Counting

This protocol can be used for accurate quantification of radioactively-labeled protein yields from EasyXpress Protein Synthesis Kit reactions. To calculate the soluble protein yield, soluble proteins can be separated from insoluble proteins by centrifugation at 12,000 x *g* for 10 minutes. Soluble protein remains in the supernatant, while insoluble protein is precipitated.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Trichloroacetic acid (TCA)
- Casein acid hydrolysate (e.g., Sigma, cat. no. A 2427)
- Glass microfibre filters (for example Whatman® GF/C)
- Vacuum manifold (e.g., Glass Microanalysis Filter Holder, Millipore cat. no. XX1002530 in combination with a vacuum pump)
- Scintillation cocktail (for example Ready Protein+™; Beckman Coulter, Inc., cat. no. 158727)
- Scintillation counter
- Acetone

Procedure

1. **Briefly vortex the protein synthesis reaction mixture and transfer a 5 µl aliquot to a test tube.**

2. **Add 3 ml of 10% (w/v) TCA solution containing 2 % casein hydrolysate.**
3. **Mix and incubate for 15 min at 90°C (during this step radiolabeled aminoacyl-tRNA as well as peptidyl-tRNA will be hydrolyzed).**
4. **Incubate for at least 30 min on ice to precipitate the synthesized proteins.**
5. **Collect the precipitate on a glass microfibre filter by using a vacuum manifold.**
   Before starting, wet the filter with a few drops of 5% (w/v) TCA.
6. **Wash the filter 3 times with 2 ml of 5% (w/v) TCA.**
7. **Dry the filter by rinsing it 2 times with 3 ml of acetone.**
8. **Transfer the filter to a scintillation vial and add an appropriate volume of scintillation cocktail.**
9. **Shake the sample gently for 1 h at room temperature (15–25°C).**
10. **Count the sample in a liquid scintillation counter.**
11. **To determine the total radioactivity added to the reactions, vortex the protein synthesis reaction mixture, transfer a 5 µl aliquot onto a filter disc placed in a scintillation vial, add scintillation cocktail and count the sample in a liquid scintillation counter.**

    Note: to determine background protein synthesis, take aliquots from the no-template control reaction and treat them as described in steps 2–10.

Calculation of protein synthesis yield:

$$\text{Percentage of } {}^{14}\text{C-Leu incorporated} = \frac{\text{counts TCA precipitation (cpm/µl)} \times 100}{\text{counts unprecipitated sample (cpm/µl)}}$$

$$\text{Yield (µg/ml)} = \frac{\%{}^{14}\text{C-Leu incorporated} \times 0.01 \times \text{conc. Leu (µM)} \times \text{mol. wt. protein (g/mol)}}{\text{Leu residues in protein} \times 1000}$$

Example

Template:	EasyXpress Positive-Control DNA
Counts:	TCA precipitated radioactivity 1730 cpm/µl
	Total radioactivity 6660 cpm/µl
Leu concentration:	1200 µM
Molecular weight:	31,489 g/mol
Leu residues:	16

% Leu incorporation = (1730/6660) x 100 = 26%

$$\text{Protein yield (µg/ml)} = \frac{26 \times 0.01 \times 1200\ \mu M \times 31{,}489\ g/mol}{16 \times 1000} = 614\ \mu g/ml$$

Appendix E: Buffers and Reagents

Buffers for purification of 6xHis-tagged proteins from *E. coli* cell lysates under native conditions using Ni-NTA Magnetic Agarose Beads

NPI-10-T (Ni-NTA Beads Binding Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
10 mM imidazole	0.68 g imidazole (MW 68.08 g/mol)
0.05% (v/v) Tween 20	5 ml of a 10% (v/v) Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

NPI-20-T (Ni-NTA Beads Wash Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
20 mM imidazole	1.36 g imidazole (MW 68.08 g/mol)
0.05% (v/v) Tween 20	5 ml of a 10% (v/v) Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

NPI-250-T (Ni-NTA Beads Elution Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
250 mM imidazole	17.0 g imidazole (MW 68.08 g/mol)
0.05% (v/v) Tween 20	5 ml of a 10% (v/v) Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Buffers for purification of 6xHis-tagged proteins from *E. coli* cell lysates under native conditions using Ni-NTA resins

NPI-10 (Ni-NTA Binding Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
10 mM imidazole	0.68 g imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

NPI-20 (Ni-NTA Wash Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
20 mM imidazole	1.36 g imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

NPI-250 (Ni-NTA Elution Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
250 mM imidazole	17.0 g imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH.

Buffers for purification of *Strep*-tagged proteins using *Strep*-Tactin Magnetic Beads

Buffer NP-T (*Strep*-Tactin Beads Wash Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
0.05% (v/v) Tween 20	5 ml of a 10% (v/v) Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Buffer NPB-T (*Strep*-Tactin Beads Elution Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
10 mM Biotin	2.44 g Biotin (e.g., Sigma cat. no. B 4501)
0.05% (v/v) Tween 20	5 ml of a 10% (v/v) Tween 20 stock solution

Adjust pH to 8.0 using NaOH.

Buffers for purification of proteins using *Strep*-Tactin Superflow

Buffer NP (*Strep*-Tactin Superflow Wash Buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)

Adjust pH to 8.0 using NaOH.

Buffer NPD (*Strep*-Tactin Superflow Elution buffer, 1 liter):

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
2.5 mM desthiobiotin	0.54 g desthiobiotin (Sigma cat. no. D 1411)

Adjust pH to 8.0 using NaOH.

***Strep*-Tactin Superflow Regeneration Buffer (1 liter):**

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
1 mM HABA	0.24 g HABA (Sigma cat. no. H 5126)

Adjust pH to 8.0 using NaOH.

Appendix F: Regeneration of *Strep*-Tactin Superflow

Strep-Tactin Superflow can be regenerated according to the following procedure. The resin can be regenerated a maximum of two times and should then be discarded.

1. **Wash the column three times with 5 column volumes *Strep*-Tactin Regeneration Buffer.**

   Strep-Tactin Regeneration Buffer contains HABA (4-hydroxyazobenzene-2-carboxylic acid). The color change from white to red indicates that the column has been regenerated by displacement of desthiobiotin.

2. **Wash the column twice with 4 column volumes of *Strep*-Tactin Superflow Lysis Buffer.**
3. **Store *Strep*-Tactin Superflow resin in *Strep*-Tactin Superflow Lysis Buffer at 4°C.**

References

1) Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning — A laboratory Manual*. 2nd Ed. Cold Spring Harbor. Cold Spring Harbor Laboratory Press.

2) Zacharias, A., Schäfer, F., Müller, S., and von Groll, U. (2004). Recombinant-protein solubility screening using the EasyXpress in vitro translation system. QIAGEN News 2004 e6. www1.qiagen.com/literature/qiagennews/weeklyArticle/04_02/e6/default.aspx

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EasyXpress pIX3.0 Vector	For protein expression in scaled-up in vitro translation reactions or *E. coli*: 25 µg vector DNA	32733
EasyXpress Protein Synthesis Mini Kit	For 20 x 50 µl reactions: *E. coli* extract, reaction buffer, RNase-Free Water, and positive-control DNA	32502
EasyXpress Protein Synthesis Maxi Kit	For reactions up to 4000 µl: 4 x 350 µl *E. coli* extract, reaction buffer, RNase-Free Water, and positive-control DNA	32506
EasyXpress Random Biotin Kit	For 20 x 50 µl reactions: *E. coli* extract, reaction buffers, RNase-Free Water, biotinyl-lysyl tRNA (Phe), and positive-control DNA	32612
Related products		
EasyXpress Site-Specific Biotin Kit	For 5 x 25 µl reactions: *E. coli* extract, reaction buffer, RNase-Free Water, biotinyl-lysyl tRNA (amber), and positive-control DNA	32602
EasyXpress Protein Synthesis Mega Kit	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o methionine, methionine, RNase-Free Water, gel-filtration columns, and reaction flasks	32516
EasyXpress NMR Protein Synthesis Kit	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o Arg, Lys, Ser, Thr, Val (supplied as individual amino acids), RNase-Free Water, gel-filtration columns, and reaction flasks	32526

Ordering Information

Product	Contents	Cat. no.
EasyXpress NMR Protein Synthesis Kit –X*	For 2 x 5 ml reactions: *E. coli* extract, amino acid mix w/o X, reaction buffers, RNase-Free Water, gel-filtration columns, and reaction flasks	Varies
EasyXpress Protein Synthesis Insect Kit	For 20 x 50 µl reactions: *Spodoptera frugiperda* insect cell extract, in vitro transcription reaction components, reaction buffers, RNase-Free Water, and positive-control DNA	32552
EasyXpress pIX4.0 Vector	25 µg vector DNA for efficient synthesis of proteins using the EasyXpress Protein Synthesis Insect Kit	32713
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Ni-NTA Superflow Columns (12 x 1.5 ml)	For 12 6xHis-tagged protein preps: 12 polypropylene columns containing 1.5 ml Ni-NTA Superflow	30622
Ni-NTA Superflow (25 ml)	25 ml nickel-charged resin (max. pressure: 140 psi)	30410
Ni-NTA Agarose (25 ml)	25 ml nickel-charged resin (max. pressure: 2.8 psi)	30210
Strep-Tactin Superflow (2 ml)	For batch and HPLC purification of *Strep*-tagged proteins: 2 ml *Strep*-Tactin-charged Superflow (max. pressure: 140 psi)	30001
Polypropylene Columns (5 ml)	50/pack, 5 ml capacity	34964
QIArack	1 rack for holding gel-filtration columns or affinity-resin filled polypropylene columns	19015

* Kits available for substitution of Ala, Cys, Asp, Glu, Phe, Gly, His, Ile, Leu, Met, Asn, Pro, Gln, Trp, and Tyr.

Ordering Information

Product	Contents	Cat. no.
Ni-NTA Magnetic Agarose Beads (2 x 1 ml)	2 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36111
Strep-Tactin Magnetic Beads (2 x 1 ml)	For micro-scale purification of *Strep*-tagged proteins: 2 x 1 ml *Strep*-Tactin-charged magnetic agarose beads (10% suspension)	36311
Factor Xa Protease*	400 units Factor Xa Protease (2 units/µl)	33223
Xa Removal Resin	2 x 2.5 ml Xa Removal Resin, 3 x 1.9 ml 1 M Tris-Cl, pH 8.0	33213
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Penta-His HRP Conjugate Kit	125 µl Penta-His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer (10x concentrate)	34460
Strep-tag Antibody (100 ug)	Mouse monoclonal antibody that recognizes the *Strep*-tag II epitope; lyophilized, for 1000 ml working solution	34850
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* Not available in all countries, please inquire.

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* Contains 5 mM each dNTP.

Notes

Notes

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