

EasyXpress[®] Protein Synthesis Handbook

For scalable cell-free expression of recombinant proteins using *E. coli* lysates

EasyXpress Protein Synthesis Kit

EasyXpress Protein Synthesis Maxi Kit

EasyXpress Random Biotin Kit



QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com .

Contents

Kit Contents	4
Storage	4
Product Use Limitations	5
Product Warranty and Satisfaction Guarantee	5
Technical Assistance	5
Safety Information	6
Quality Control	6
Introduction	7
The EasyXpress System	8
Principle and Procedure	11
Expression Templates	12
Purification of In Vitro-Synthesized Proteins	14
Cotranslational Biotin Labeling of Proteins	16
Protocols	
■ Cell-Free Expression Using a PCR Product as Template	18
■ Cell-Free Expression Using Plasmid DNA as Template	22
■ Scaling Up Cell-Free Expression Reactions Using the EasyXpress Protein Synthesis Maxi Kit	26
■ Scaling Down Cell-Free Expression Reactions Using the EasyXpress Protein Synthesis Maxi Kit	28
Troubleshooting Guide	31
References	35
Appendix A: Purification of In Vitro Synthesized Proteins	36
Appendix B: Optimization of EasyXpress Small-Scale Reactions	43
Appendix C: Incorporating Radioactive Labels into Proteins for Quantification	45
Appendix D: Buffers and Reagents	49
Appendix E: Regeneration of Strep-Tactin Superflow	51
Ordering Information	52

Kit Contents

EasyXpress Protein Synthesis Kit			Maxi Kit
Number of reactions	5 x 50 μ l	20 x 50 μ l	4 x 1 ml
Cat. no.	32501	32502	32506
EasyXpress <i>E. coli</i> Extract (colorless snap-cap)	5 x 17.5 μ l	20 x 17.5 μ l	4 x 350 μ l
EasyXpress Reaction Buffer (blue screw-cap)	1 x 100 μ l	1 x 400 μ l	4 x 400 μ l
RNase-Free Water (colorless screw-cap)	1 x 1.9 ml	1 x 1.9 ml	1 x 1.9 ml
EasyXpress Positive-Control DNA (yellow screw-cap)	1 x 12.5 μ l	1 x 50 μ l	1 x 50 μ l
Handbook	1	1	1

EasyXpress Random Biotin Kit	For 60 x 50 μ l reactions
Cat. no.	32612
EasyXpress Biotinyl-Lysyl tRNA (Phe)	4 x 15 μ l
Product Sheet	1

Storage

EasyXpress Protein Synthesis 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.

The **EasyXpress Random Biotin Kit** is shipped on dry ice and should be stored at -70°C . 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 four times.

When stored under the above conditions and handled correctly, all kits can be kept for at least 12 months without showing any reduction in performance.

Product Use Limitations

EasyXpress Kits are developed, designed, and sold for research purposes only. They are 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.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

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. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

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 back cover or visit www.qiagen.com).

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 sample and assay technologies 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 see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

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.

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. Modern protein engineering methods, which include cloning of DNA sequences and the *in vivo* expression of genes, allow production of specific proteins in large amounts and also production of 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 [IVT] systems), which are often seen as a very attractive alternative to classical *in vivo* expression systems. Cell-free expression generates proteins by coupled or successive transcription and translation in cell-free extracts of prokaryotic or eukaryotic cells. The advantages of cell-free expression 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 cofactors). Compared with current cloning techniques, another important advantage offered by a cell-free expression system is the possibility of using PCR products as templates for protein synthesis. This greatly accelerates the protein production process, because no cloning steps are required. Moreover, there is no need for any specialized equipment: only an incubator, pipette, and reaction tubes are needed.

Proteins produced by cell-free expression can be used for the same wide variety of downstream applications as *in-vivo* produced proteins, including activity assays, structural and functional 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 in *E. coli*, 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.

However, obtaining sufficient amounts of soluble, active protein is a bottleneck in many projects. For proteins that are sparingly soluble, addition of one or more affinity tags not only facilitates purification, but can also increase expression and/or solubility and therefore yield. Because it is difficult to predict the effect that adding a tag will have on the expression of a given protein, generating and analyzing a range of expression constructs in which the added tags are systematically re-arranged gives the best chance of finding the optimal construct.

Identification of optimal constructs in a single day

The PCR-based EasyXpress Linear Template Kit Plus (cat. no. 32723) can be used to generate a range of transcription templates that encode target proteins with varying combinations of 6xHis and *Strep*-tag affinity tags (Figure 1). The PCR products are generated in a 2-step PCR procedure that uses target-specific primers (supplied by the user) and specially designed tag-encoding primers, which encode regulatory elements that optimize transcription and translation in cell-free expression reactions.

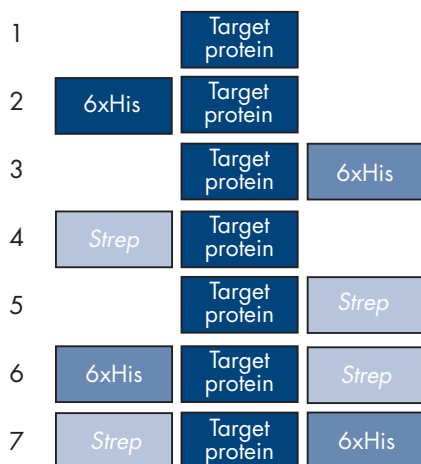


Figure 1. The variety of constructs whose expression can be tested using the Qproteome Linear Template and Protein Synthesis Kits.

The screening of such constructs in small-scale cell-free expression (IVT) reactions using *E. coli*-based EasyXpress Protein Synthesis Kits (or the insect-cell based EasyXpress Insect Kit II) saves a significant amount of time. The analysis of cell-free expressed proteins by western blotting with tag-specific antibodies enables the identification of the construct or reaction conditions that provide the highest ratio of soluble protein. This procedure also enables a number of different mutation or truncation forms to be tested to find the best-expressing construct (Figure 2). Expression efficiencies obtained using in vitro systems correlate well with those seen in in vivo systems, meaning that the best-expressing construct identified in the screening procedure will usually deliver optimal yields in scaled-up in vivo expression.

“We tested the major IVT kits on the market and found that the new EasyXpress kits from QIAGEN deliver excellent results. Because of the good correlation between cell-free and cell-based expression in *E. coli*, this product is an ideal tool for protein expression screening.”

Thorsten Lamla, Ph.D., Research Scientist, NBE Laboratory, Boehringer Ingelheim

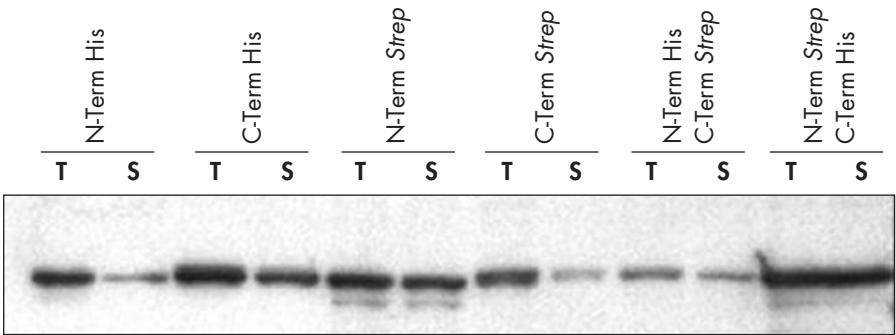


Figure 2. Expression analysis reveals the optimal construct. PCR products expressing IRAK4 and carrying the indicated tag(s) were added to small-scale *E. coli* IVT reactions. Samples of total protein (T, complete reaction) and soluble protein (S, supernatant of complete reaction after centrifugation) were separated by SDS-PAGE, transferred to a membrane, and visualized using a mixture of His- and Strep-tag antibodies and chemiluminescent detection.

Scaling up cell-free protein synthesis reactions

Once the optimal construct has been identified it can be directly cloned into the pIX 3.0 vector for scaled up *in vivo* or *in vitro* expression in *E. coli*. For structural biologists who want to express large amounts of protein containing isotopically labeled or modified amino acids, QIAGEN offers the EasyXpress NMR Protein Synthesis Kit (cat. no. 32526) and EasyXpress Protein Synthesis Mega Kit (cat. no. 32516), which enable up to 5 mg cotranslationally labeled protein to be synthesized and purified in just 5 hours. To learn more about the EasyXpress system, visit www.qiagen.com/easyxpress or see references 1–2.

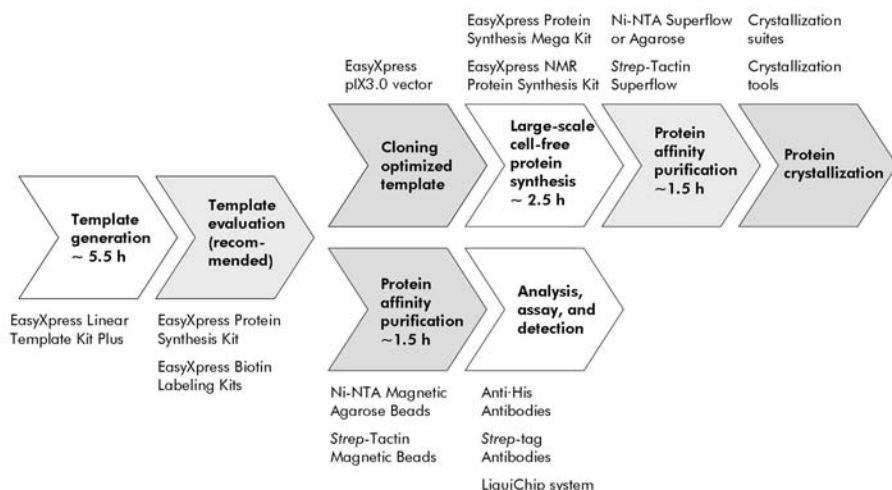


Figure 3. Expression, purification, detection, and analysis form an integrated workflow chain.

Expression testing of QIAGENs Expression Constructs

QIAGENs are available from QIAGEN as genome-wide expression-ready vectors. These are optimized synthetic human genes, ready-cloned into expression vectors covering all 35,000 human ORFs and are now available from the GeneGlobe database (www.geneglobe.com). The QIAGENs Expression Constructs are optimized with respect to, among others, codon usage of host system, mRNA stability and secondary structures, and G/C content. Initial expression testing of QIAGENs Expression Constructs optimized for *E. coli* expression can be very quickly performed using the EasyXpress Protein Synthesis Kit (5) (cat. no. 32501).

Principle and Procedure

The highly productive *E. coli* cell lysates in *E. coli*-based EasyXpress Protein Synthesis Kits are continually being optimized in terms of yields. Currently in the fifth generation of their development, recent improvements have led to improved performance and yields of functional protein, especially when PCR products are used as templates.

The lysates contain all translational machinery components (ribosomes, ribosomal factors, tRNAs, aminoacyl-tRNA synthetases, etc.) as well as T7 and *E. coli* RNA polymerases. They use a coupled transcription–translation system enabling the expression of 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 4). Using EasyXpress Protein Synthesis Kits, up to 1 mg/ml biologically active protein can be synthesized within 1 hour (2). 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). Large- and small-scale protocols are available in this handbook. The amount of protein synthesized increases linearly with reaction volume.

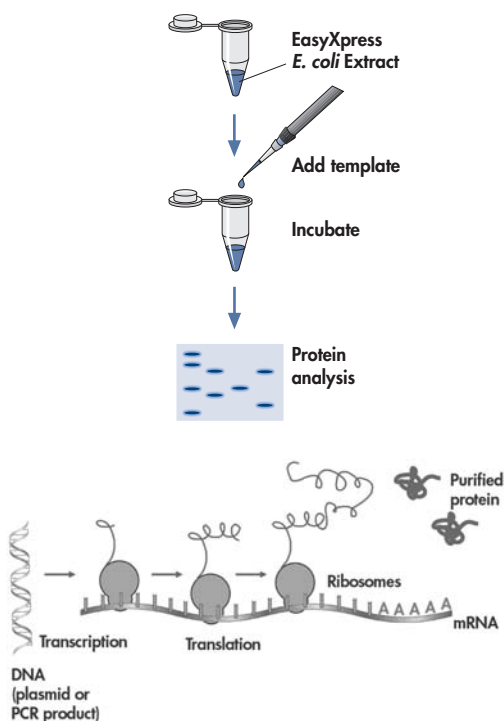


Figure 4. Schematic showing cell-free expression of proteins.

Expression Templates

EasyXpress Kits can be used to express proteins from a variety of DNA templates that contain a T7 or a strong *E. coli* promoter upstream of the coding sequence. Further prerequisites of DNA-templates are an efficient ribosome binding site (RBS) with a spacing sequence of 5–7 bp to the translational start point (preferably AUG) and an untranslated region (UTR) downstream of the stop codon (Figure 5).

In addition, we have incorporated elements in our expression constructs to increase stability and translational efficiency that are present in many standard expression vectors. Stem-loop structures, recommended for stabilizing the mRNA transcript against nucleases, form at the mRNA 5' and 3' termini, thereby increasing the efficiency of expression. Translational enhancers like epsilon sequence derived from phage T7 gene 10 have been reported to enormously improve protein synthesis (3). Suitable DNA templates include linear DNA (e.g. PCR products) and supercoiled plasmids.

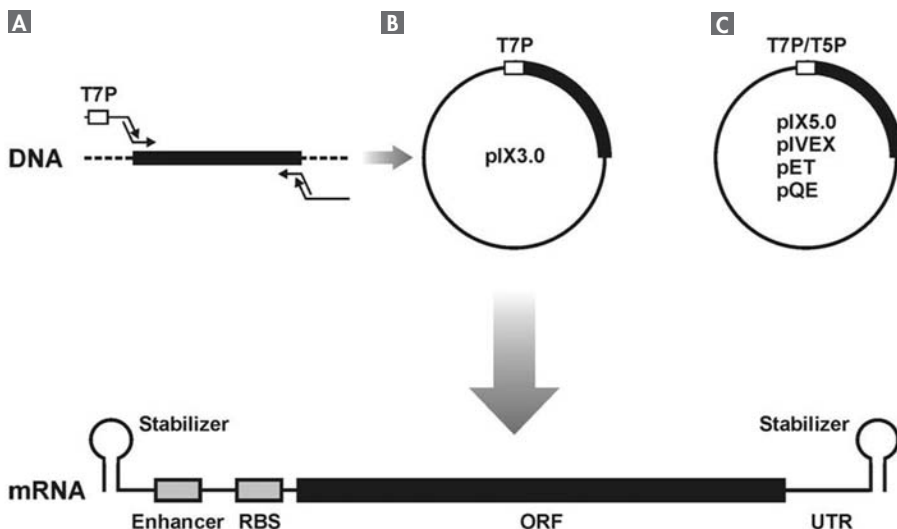


Figure 5. Different types of DNA templates compatible with the EasyXpress System (top) and optimal organization of an mRNA for efficient cell-free expression (bottom). **A** Linear template generated with the EasyXpress Linear Template Kit Plus. **B** Linear template subcloned into vector pIX3.0. **C** Other vectors containing a T7 or T5 promoter. Abbreviations: **T7P**: T7 promoter, **T5P**: T5 promoter, **RBS**: ribosome binding site, **ORF**: open reading frame, **UTR**: untranslated region.

PCR products

If PCR products are added to cell-free expression reactions, we recommend that they are generated using the EasyXpress Linear Template Kit Plus (cat. no. 32723). PCR products can be added directly to cell-free expression reactions without further cleanup. The amount of PCR product added to each 50 µl cell-free expression reaction should be 0.7–1.0 µg. In addition, XE-Solution (provided with the EasyXpress Linear Template Kit Plus) should be added to cell-free expression reactions to increase the efficiency of transcription and translation. XE solution is effective only with the adapter primers supplied with the EasyXpress Linear Template Kit Plus.

If PCR products need to be concentrated, we recommend using the QIAGEN MinElute® PCR Purification Kit (cat. no. 28004).

The section “Generating PCR Products for In Vitro Translation” in the *EasyXpress Linear Template Kit Handbook* gives comprehensive and detailed information on producing PCR products suitable for use as expression constructs with EasyXpress Protein Synthesis Kits. The PCR products generated using the EasyXpress Linear Template Kit can be easily and quickly cloned into the pIX3.0 expression vector for scaled up expression in large-scale cell-free expression reactions or *E. coli* cells (Figure 5B).

Plasmid DNA

Plasmids containing a T7 promoter — such as pIX5.0 (RiNA GmbH) and the pET (Novagen) and pIVEX plasmid series (Roche) — are a suitable basis for generating expression constructs. However, use of vectors is not restricted to T7 promoter-based constructs. QIAGEN’s T5 promoter-based pQE vectors have been used successfully for in vitro protein synthesis using EasyXpress *E. coli*-based kits. Highest 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 (4) may be sufficiently pure, but DNA must be free of RNases. The concentration of plasmid DNA in each 50 µl cell-free expression 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 (T5), the higher concentration of 10 nM should be used.

QIAGENes Expression Constructs can be directly evaluated using EasyXpress *E. coli* lysates. QIAGENes Expression Constructs are ready-to-use plasmids carrying expression-optimized synthetic genes under the control of a T7 promoter coding for an N-terminally His-tagged protein. They provide an Amber stop codon (UAG) at the C-terminus for site-specific incorporation of one biotin molecule per protein molecule using the EasyXpress Site-Specific Biotin Kit. Biotinylated proteins can be used for protein-protein interaction studies and immobilization on streptavidin supports. See the *EasyXpress Site-Specific Biotinylated Protein Synthesis Handbook* for more details.

Purification of In Vitro-Synthesized Proteins

For many applications where labeled proteins are used (e.g., BIACORE® and xMAP™ 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 6).

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.

The NTA ligand, which has four chelation sites for nickel ions, binds nickel more tightly than metal-chelating purification systems such as resins with IDA ligands 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 36.

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 ($K_d = 1 \mu\text{M}$) 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.

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.

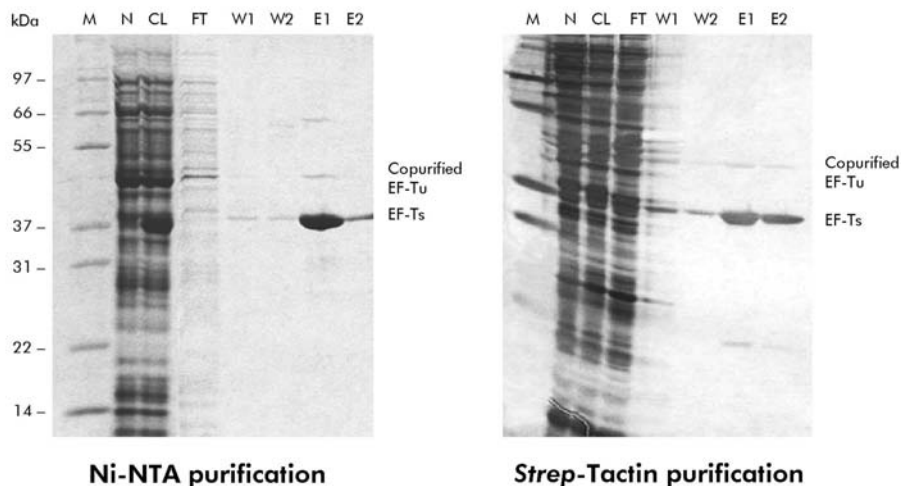


Figure 6. Highly efficient purification of active proteins from cell-free expression reactions. **A** 6xHis-tagged or **B** *Strep*-tagged *E. coli* elongation factor-Ts was purified under native conditions from 50 μ l EasyXpress Protein Synthesis Mini Kit cell-free expression reactions. Ni-NTA Magnetic Agarose Bead or *Strep*-Tactin Magnetic Bead suspension (150 μ l) was added to the crude lysate to bind protein. The beads were washed with 500 μ l wash buffer to remove contaminants and pure 6xHis- or *Strep*-tagged protein was eluted in two 50 μ l 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.

Cotranslational Biotin Labeling of Proteins

Labeling proteins can dramatically simplify their study. Adding a specific label or tag to different proteins enables their immobilization or detection using a common procedure or reagent.

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

QIAGEN offers the EasyXpress Random Biotin Kit for random cotranslational non-radioactive labeling of proteins. Using cell-free expression in *E. coli*- or insect-cell extracts, biotin moieties are incorporated into recombinant proteins with high efficiency. 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 comprises a synthetic tRNA aminoacylated with lysine labeled at the epsilon position with biotin and carrying a phenylalanine GAA anticodon. This tRNA directs the incorporation of a biotin residue at phenylalanine UUC codons (Figure 7). 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 1). During protein synthesis, either biotin-labeled lysine or unlabeled phenylalanine (from the reaction buffer) is incorporated. Biotin incorporation occurs statistically, but with high efficiency (Figure 8). 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 1. 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

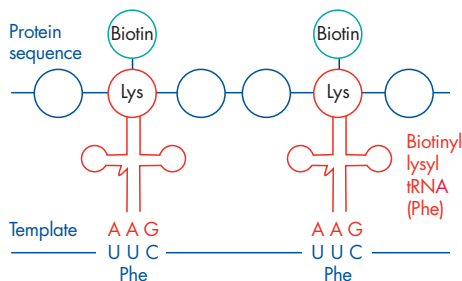


Figure 7. Schematic representation of biotin incorporation using the EasyXpress Random Biotin Kit.

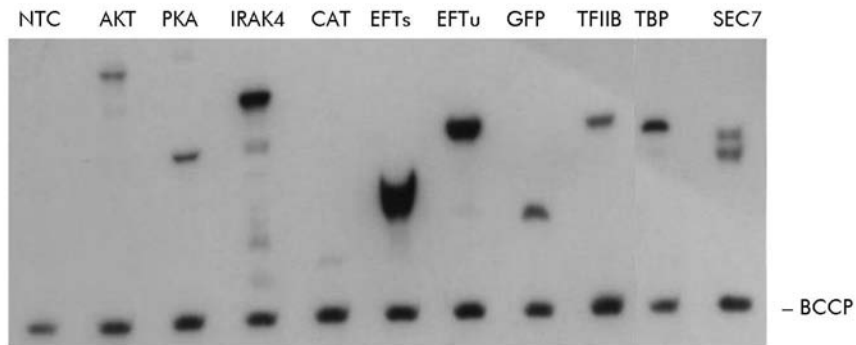


Figure 8. Synthesis of biotinylated recombinant proteins using the QIAGEN EasyXpress Random Biotin Kit, which inserts biotinylated lysine residues at phenylalanine UUC codons. BCCP: endogenous *E. coli* biotin carboxyl carrier protein; NTC: no template control; AKT: human RAC-alpha serine/threonine kinase (AKT1); 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; TFII: human Transcription Factor IIB; TBP: human TATA-box binding protein; SEC7: human cytohesin-1 SEC7phs domain.

Protocol: Cell-Free Expression Using a PCR Product as Template

This protocol is suitable for production of recombinant proteins using PCR-generated DNA templates and EasyXpress Protein Synthesis 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 cell-free expression reactions without further cleanup. To facilitate sample analysis, proteins can be labeled with biotin using the EasyXpress Random Biotin Kit.

Reactions can be easily scaled down to as little as 5 μ l for high-throughput screening in 96-well microplate format. The reagent volumes given in Table 2 should be adjusted accordingly. We recommend using polypropylene 96-well plates (e.g., Greiner Bio-One cat. no. 650201) for reaction volumes >25 μ l and 96-well thin-wall polycarbonate PCR plates (e.g., Corning Incorporated, cat. no. 6513) for reaction volumes <25 μ l. During the cell-free expression reaction in an incubator or on a PCR cycler, microplates and PCR plates should be sealed with a tape sheet to avoid evaporation.

Materials and reagents to be supplied by user

- PCR template encoding the protein of interest and PCR template derived from control DNA (see page 13)
- Thermomixer® (Eppendorf, Hamburg, Germany), water-bath, or heating block

Important general points before starting

- The cell-free expression 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. This PCR product 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.
- The majority of low molecular weight components in *E. coli* lysates are separated during preparation of the *E. coli* Extract in EasyXpress Kits. Therefore, if the protein to be expressed requires a cofactor or coenzyme (e.g., metal ions or vitamin) to fold or function properly, this factor should be added to the reaction (see Appendix B).

Additional points to consider when producing biotinylated proteins

- For multiple reactions, prepare a master mix without template and Biotinyl-Lysyl-tRNA, transfer aliquots to reaction tubes, and initiate protein synthesis by adding template and Biotinyl-Lysyl-tRNA.
- 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 reaction mix as recommended in the protocol.
- 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 for cotranslational labeling, thaw EasyXpress Biotinyl-Lysyl tRNA (Phe) (orange screw-cap) on ice.**

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

Table 2 provides a pipetting scheme for EasyXpress protein synthesis reactions using PCR products.

2. **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.**
3. **Mix and centrifuge briefly to collect reactions in the bottom of the tubes.**

Table 2. 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
<i>E. coli</i> Extract	17.5 μ l	17.5 μ l	17.5 μ l
RNase-free water	Add to 50 μ l	Add to 50 μ l	Add to 50 μ l
XE-Solution*	2 μ l	2 μ l	2 μ l
DNA template from second PCR	approx. 0.7 μ g	7 μ l	–
EasyXpress Reaction Buffer	20 μ l	20 μ l	20 μ l
When producing randomly biotinylated proteins:			
Biotinyl-Lysyl-tRNA (Phe)	1–3 μ l [†]	1–3 μ l [†]	1–3 μ l [†]
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.

[†] For routine applications, add 1 μ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each 50 μ l translation reaction. To enhance signal intensity in detection procedures, the proportion of incorporated biotin can be increased by adding up to 3 μ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) per reaction.

- For each reaction add 2 μ l XE-Solution to 0.7 μ g DNA (~7 μ l) from the second PCR in a separate tube and mix by pipetting up and down. For the positive-control reaction add 2 μ l XE-Solution to 7 μ l 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 μ l XE-Solution and 7 μ l RNase-free water.

- Add the XE-Solution-PCR product mix from step 4 to each reaction, mix, and centrifuge briefly to collect reactions in the bottom of the tubes.
- Add 20 μ l of EasyXpress Reaction Buffer to the mix from step 5, mix, and centrifuge briefly to collect reactions in the bottom of the tubes.

- 7. If producing biotinylated proteins using the EasyXpress Random Biotin Kit, add 1–3 μ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each reaction, mix, and centrifuge briefly to collect reactions in the bottom of the tubes. Otherwise proceed to step 8.**

For routine applications, add 1 μ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each 50 μ l translation reaction. To enhance signal intensity in detection procedures, the proportion of incorporated biotin can be increased by adding up to 3 μ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) per reaction.

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

- 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.**

Well-expressed proteins can be visualized by separation using SDS-PAGE and Coomassie Staining. Weakly expressed proteins can be visualized by separation using SDS-PAGE, blotting onto a cellulose membrane, and immunodetection using a protein- or tag-specific antibody.

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 μ l of the cell-free expression reaction is sufficient for one gel lane.

Protocol: Cell-Free Expression Using Plasmid DNA as Template

This protocol is suitable for production of recombinant proteins using plasmid DNA templates and EasyXpress Protein Synthesis Kits. To facilitate sample analysis, proteins can be labeled with biotin using the EasyXpress Random Biotin Kit.

Reactions can be easily scaled down to as little as 5 μ l for high-throughput screening in 96-well microplate format (see protocol, page 28). The reagent volumes given in Table 3 should be adjusted accordingly. We recommend using polypropylene 96-well plates (e.g., Greiner Bio-One cat. no. 650201) for reaction volumes >25 μ l and 96-well thin-wall polycarbonate PCR plates (e.g., Corning Incorporated, cat. no. 6513) for reaction volumes <25 μ l. During the cell-free expression reaction in an incubator or on a PCR cycler, microplates and PCR plates should be sealed with a tape sheet to avoid evaporation.

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 cell-free expression 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.
- The recommended incubation temperature for protein synthesis is 37°C, but lower incubation temperatures may improve protein solubility in some cases.

- If the plasmid DNA template contains IPTG-inducible promoter systems (e.g., T5 or T7) add IPTG (final concentration 1 mM) to the translation reaction.
- The majority of low molecular weight components in *E. coli* lysates are separated during preparation of the *E. coli* Extract in EasyXpress Kits. Therefore, if the protein to be expressed requires a cofactor or coenzyme (e.g., metal ions or vitamin) to fold or function properly, this factor should be added to the reaction (see Appendix B).

Additional points to consider when producing biotinylated proteins

- 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 template and the Biotinyl-Lysyl-tRNA.
- 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 reaction mix as recommended in the protocol.
- 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 for cotranslational labeling, thaw EasyXpress Biotinyl-Lysyl tRNA (Phe) (orange screw-cap) on ice.**

Gently vortex all components and spin down before use.

Table 3 provides a pipetting scheme for EasyXpress protein synthesis using plasmid DNA.

2. **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.**
3. **Mix and centrifuge briefly to collect reactions in the bottom of the tubes.**
4. **Add 5–10 nM (~ 0.5 –1 μg) plasmid DNA to each reaction, mix, and centrifuge briefly to collect reactions in the bottom of the tubes.**

Table 3. 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
<i>E. coli</i> 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)	1–3 µl†	1–3 µl†	1–3 µl†
Total	50 µl	50 µl	50 µl

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

† For routine applications, add 1 µl EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each 50 µl translation reaction. To enhance signal intensity in detection procedures, the proportion of incorporated biotin can be increased by adding up to 3 µl EasyXpress Biotinyl-Lysyl-tRNA (Phe) per reaction.

5. Add 20 µl of EasyXpress Reaction Buffer to the mix from step 4, mix, and centrifuge briefly to collect reactions in the bottom of the tubes.
6. If producing biotinylated proteins using the EasyXpress Random Biotin Kit, add 1–3 µl EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each reaction, mix, and centrifuge briefly to collect reactions in the bottom of the tubes. Otherwise proceed to step 7.

For routine applications, add 1 µl EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each 50 µl translation reaction. To enhance signal intensity in detection procedures, the proportion of incorporated biotin can be increased by adding up to 3 µl EasyXpress Biotinyl-Lysyl-tRNA (Phe) per reaction.

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

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.

Well-expressed proteins can be visualized by separation using SDS-PAGE and Coomassie Staining. Weakly expressed proteins can be visualized by separation using SDS-PAGE, blotting onto a cellulose membrane, and immunodetection using a protein- or tag-specific antibody.

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 μ l of the cell-free expression reaction is sufficient for one gel lane.

Protocol: Scaling Up Cell-Free Expression 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 or down accordingly. Smaller-volume cell-free expression reactions (50–500 μ l) should be performed in 1.5 ml reaction tubes. Translation reactions >500 μ l should be incubated in suitable reaction tubes (e.g., a 15 ml polypropylene tube).

Materials to be supplied by user

- Plasmid DNA template encoding the protein of interest
- Thermomixer (Eppendorf, Hamburg, Germany), incubator, or water-bath
- Sterile 1.5 ml or 15 ml reaction tubes

Important points before starting

- The cell-free expression 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 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 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.
- If the plasmid DNA template contains IPTG-inducible promoter systems (e.g., T5 or T7) add IPTG (final concentration 1 mM) to the translation reaction.
- The majority of low molecular weight components in *E. coli* lysates are separated during preparation of the *E. coli* Extract in EasyXpress Kits. Therefore, if the protein to be expressed requires a cofactor or coenzyme (e.g., metal ions or vitamin) to fold or function properly, this factor should be added to the reaction (see Appendix B).

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).

Gently vortex all components and spin down before use.

2. Table 4 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 15 ml polypropylene tube containing *E. coli* Extract (350 µl for a 1 ml reaction).
3. Mix and centrifuge briefly to collect reactions in the bottom of the tubes.
4. Add 5–10 nM (~10–20 µg) plasmid DNA to each reaction, mix, and centrifuge briefly to collect reactions in the bottom of the tubes.
5. Add 400 µl of Reaction Buffer to the mix from step 4, mix, and centrifuge briefly to collect reactions in the bottom of the tubes.
6. 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.

7. Proceed with sample analysis.

Well-expressed proteins can be visualized by separation using SDS-PAGE and Coomassie Staining. Weakly expressed proteins can be visualized by separation using SDS-PAGE, blotting onto a cellulose membrane, and immunodetection using a protein- or tag-specific antibody.

Table 4. 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
<i>E. coli</i> Extract	350 µl	17.5 µl	17.5 µl
RNase-free water	Add to 1 ml	10 µl	12.5 µl
DNA template	5–10 nM	2.5 µl	–
EasyXpress Reaction Buffer	400 µl	20 µl	20 µl
Total	1000 µl	50 µl	50 µl

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

Protocol: Scaling Down Cell-Free Expression Reactions Using the EasyXpress Protein Synthesis Maxi Kit

This protocol is suitable for the high-throughput in vitro production of recombinant proteins using plasmid DNA templates and the EasyXpress Protein Synthesis Maxi Kit in a 96-well microplate (40 μ l reaction volume). To facilitate sample analysis, proteins can be labeled with biotin using the EasyXpress Random Biotin Kit.

Materials to be supplied by user

- Plasmid DNA template encoding the protein of interest
- Heatable microplate incubator
- Polypropylene 96-well plate (e.g., Greiner Bio-One, cat. no. 650201)
- Sealing tape for microplates (e.g., Tape Pads, QIAGEN cat. no. 19570)
- Sterile 15 ml polypropylene reaction tube

Important points before starting

- The cell-free expression system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- 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 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 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.
- If the plasmid DNA template contains IPTG-inducible promoter systems (e.g., T5 or T7) add IPTG (final concentration 1 mM) to the translation reaction.
- The majority of low molecular weight components in *E. coli* lysates are separated during preparation of the *E. coli* Extract in EasyXpress Kits. Therefore, if the protein to be expressed requires a cofactor or coenzyme (e.g., metal ions or vitamin) to fold or function properly, this factor should be added to the reaction (see Appendix B).

Additional points to consider when producing biotinylated proteins

- For multiple reactions, prepare a master mix without template and Biotinyl-Lysyl-tRNA. Initiate protein synthesis by adding the master mix to the template solution(s) containing Biotinyl-Lysyl-tRNA.
- 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. Add Biotinyl-Lysyl-tRNA to the template solution(s) as recommended in the protocol.
- 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 *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 ($15-25^{\circ}\text{C}$). If using the EasyXpress Random Biotin Kit for cotranslational labeling, thaw EasyXpress Biotinyl-Lysyl tRNA (Phe) (orange screw-cap) on ice.**

Gently vortex all components and spin down before use.

2. **Add 10 μl diluted template to each well of the microplate. Optional: to generate biotinylated proteins add 0.8–2.4 μl Biotinyl-Lysyl-tRNA to each well.**
3. **Prepare an EasyXpress Extract Master-Mix on ice by mixing 14 x X μl EasyXpress *E. coli* Extract with 16 x X μl EasyXpress Reaction Buffer (X = number of wells to be used).**

For example, to prepare a master mix for 50 wells, mix 14 x 50 (= 700 μl) EasyXpress *E. coli* Extract with 16 x 50 (= 800 μl) EasyXpress Reaction Buffer. Vortex and centrifuge briefly.

4. **Start the translation reactions by adding 30 μl master mix from step 3 to each well containing template.**
5. **Seal the microplate with tape and place on an incubator at 37°C for 1 h, if possible with shaking.**

Component	Samples	Positive control*	No template control
RNase-free water	Add to 10 μ l	Add to 10 μ l	Add to 10 μ l
DNA template (plasmid)	5–10 nM	2 μ l	–
When producing randomly biotinylated proteins:			
Biotinyl-Lysyl-tRNA (Phe)	0.6–2.4 μ l [†]	0.6–2.4 μ l [†]	0.6–2.4 μ l [†]
Total	10 μl	10 μl	10 μl

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

[†] For routine applications, add 0.6 μ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) to each 40 μ l translation reaction. To enhance signal intensity in detection procedures, the proportion of incorporated biotin can be increased by adding up to 2.4 μ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) per reaction.

6. Proceed with sample analysis.

Well-expressed proteins can be visualized by separation using SDS-PAGE and Coomassie Staining. Weakly expressed proteins can be visualized by separation using SDS-PAGE, blotting onto a cellulose membrane, and immunodetection using a protein- or tag-specific antibody.

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 μ l of the cell-free expression reaction is sufficient for one gel lane.

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

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 <i>E. coli</i> 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 cell-free expression. |
| 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 cell-free expression 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. |

Comments and suggestions

- | | |
|---|---|
| c) In vitro transcription or cell-free expression is disrupted by expressed protein | Express control protein in the presence of the target protein. If expression of control protein is strongly* 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 | <p>Include a 6xHis-tag coding sequence at the 5' end of the protein coding sequence.</p> <p>If the protein to be expressed already contains a tag, move the tag to the opposite terminus.</p> <p>PCR templates generated using the EasyXpress Linear Template Kit Plus code for optimized and cleavable initiator mRNA sequences, and we therefore recommend using this kit to generate expression templates.</p> |

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 | <p>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.</p> <p>Determine the optimal amount of DNA template used in the cell-free expression by titration.</p> |
| c) If protein is expressed with a tag, the tag may have 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 (5). |

* The expression level of the control protein may be lower than normal as the transcription and translation machinery is being shared with the target protein template.

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.

PCR templates generated using the EasyXpress Linear Template Kit Plus code for optimized and cleavable initiator mRNA sequences, and we therefore recommend using this kit to generate expression templates. |
| e) Plasmid DNA template contains <i>E. coli</i> 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 | <i>E. coli</i> 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. See Appendix B for a list of additives compatible with EasyXpress Kits. |
| c) Disulfide bonds required for activity and correct folding | Perform expression reaction in the presence of Redox buffer (see Table 6, Appendix B) |

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. Further methods for improving protein solubility can be found in Appendix B. |
|--------------------------|--|

Expressed protein is degraded or fragmented

- | | |
|---|---|
| a) Premature termination of translation | Check reading frame of the target sequence for mutations that might produce a stop codon. |
|---|---|

- | | |
|---|---|
| b) Initiation of translation within the protein coding sequence | The cause of an internal initiation of translation is usually the presence of an <i>E. coli</i> ribosome binding site the correct distance away from a methionine codon. This can especially be a problem in heterologous expression (e.g., of human genes) because the coding sequence is not optimized for prokaryotic systems. For efficient heterologous expression, consider using QIAGEN Expression Constructs <i>E. coli</i> , whose protein coding sequences are optimized for expression in <i>E. coli</i> .

Check reading frame of the target sequence for mutations that might produce a start codon. |
| c) Protein is degraded by proteases | Add protease inhibitors to the cell-free expression reaction. |

Poor incorporation of biotin/weak or no 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 reaction mix as recommended in the protocol. |
| c) 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. |
| d) Detection reagents are not functioning correctly | 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. |

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Cited references

- 1) The key to getting more protein — Expression screening. QIAGEN News 2007 e3. www.qiagen.com/literature/qiagennews/weeklyArticle/07_05/e04/default.aspx
- 2) Gourdon, P. et al. (2008). Optimized in vitro and in vivo expression of proteorhodopsin: a seven-transmembrane proton pump. *Prot. Expr. Purif.* **58**, 103.
- 3) O'Connor, M., and Dahlberg, A.E. (2001) Enhancement of translation by the epsilon element is independent of the sequence of the 460 region of 16S rRNA. *Nucleic Acids Res.* **29**, 1420.
- 4) Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning — A laboratory Manual*. 2nd Ed. Cold Spring Harbor. Cold Spring Harbor Laboratory Press.
- 5) Zacharias, A., Schäfer, F., Müller, S., and von Groll, U. (2004). Recombinant-protein solubility screening using the EasyXpress cell-free expression system. QIAGEN News 2004 e6. www.qiagen.com/literature/qiagennews/weeklyArticle/04_02/e6/default.aspx

Appendix A: Purification of In Vitro Synthesized Proteins

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

This protocol can be used to efficiently purify 6xHis-tagged proteins under native conditions from 50 µl EasyXpress cell-free expression 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 49.

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 100 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 is 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 μ l 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 μ l cell-free expression 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 μ l 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 μ l 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 under native conditions from 40 µl EasyXpress cell-free expression 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 49.

Procedure

1. **Resuspend Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately pipet 120 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 100 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 is 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 μ l 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 40 μ l cell-free expression 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 320 μ l 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 μ l 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 under native conditions from 1 ml EasyXpress cell-free expression 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 50.

Procedure

1. Pipet 2 ml Buffer NPI-10 into a 14 ml tube.
2. Pipet 500 μ l of 50% Ni-NTA Superflow slurry into the tube containing binding buffer. Pipet 1 ml cell-free expression 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 20–40 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 μ l EasyXpress cell-free expression 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 50.

Procedure

1. Resuspend *Strep-Tactin* Magnetic Beads by vortexing for 2 s and then immediately pipet 200 μ l 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 μ l 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 μ l cell-free expression 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 μ l 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 μ l 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 cell-free expression 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/cell-free expression 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 51.

Procedure

1. **Pipet eluates or 1 ml cell-free expression 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 51.

Appendix B: 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 tables below give some examples of reagents and reaction conditions that may lead to improved results with regard to protein solubility and/or yield.

It may be possible to combine optimized parameters (e.g., incubation at 32°C with 2% glycerol) to further optimize expression, but such enhancements may not always be additive and their effects must be determined empirically.

If performing optimization trials using additives, the total volume of the reaction can be increased by 10% (i.e., from 50 to 55 µl) without any significant impact on the basal rate of protein synthesis.

Table 5. Addition of metal ion cofactors to EasyXpress *E. coli*-based protein synthesis reactions

Cofactor (salt)	Compatible concentration*	Cofactor (salt)	Compatible concentration*
Ca ²⁺ (CaCl ₂)	20 µM	Mo ⁶⁺ (Na ₂ MoO ₄)	375 µM
Co ²⁺ (CoSO ₄)	375 µM	Se ⁴⁺ (Na ₂ SeO ₃)	1 µM
Cu ²⁺ (CuCl ₂)	90 µM	W ⁶⁺ (Na ₂ WO ₄)	1 µM
Fe ²⁺ (FeSO ₄)	375 µM	Ni ²⁺ (NiSO ₄)	5 µM
Mn ²⁺ (MnCl ₂)	45 µM	Zn ²⁺ (ZnCl ₂)	500 µM

* This is the highest concentration tested that had no significant effect on protein synthesis in trials expressing the control protein EF-Ts (His). It is possible that higher concentrations can be added without compromising protein synthesis.

Table 6. Factors to consider when optimizing EasyXpress E. coli-based protein synthesis reactions.

Reaction condition	Range of conditions evaluated	Recommended starting point for optimization
Temperature	30–37°C	30°C
Incubation time	1–3 h	1.5 h
Reaction dilution	1.1x to 1.6x (add 5 – 30 µl water to reaction)	1.4x (add 20 µl water to reaction)
Template concentration	0.2 – 2 µg DNA per 50 µl reaction	1 µg per 50 µl reaction
Presence of IPTG (for IPTG-inducible plasmids)		1 mM final concentration
Hydroxy-ectoine	0.1–1 M	0.5 M
D-Sorbitol	0.1–1 M	0.5 M
Glycine Betaine	0.1–1 M	0.5 M
L-Carnitine	0.1–1 M	0.5 M
Detergents*	0.05–1% (v/v)	0.5% (v/v)
Glycerol	Up to 3% (v/v)	1.5% (v/v)
Presence of cofactors (metal ions)	See Table 5 above	
Redox buffer (20x) = Oxidized + Reduced glutathione = 120 mM GSSG + 12 mM GSH	0.5x – 1.5x	1x
pH adjustment†		
Acidic buffer (0.5 M MES, pH 5.5, adjusted with KOH)	Up to 11 µl per 50 µl reaction (pH 6.5–7.4)	
Basic buffer (0.25 M KOH)	Up to 6 µl per 50 µl reaction (pH 7.4–8.0)	

* Some detergents may reduce efficiency of protein expression.

† Add MES or KOH to EasyXpress Reaction Buffer and reduce water added to reaction accordingly.

Appendix C: 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 7 and 8 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.
3. Mix and centrifuge briefly to collect reactions in the bottom of the tubes.
4. If using plasmid DNA as a template, proceed using step 4a. If using a PCR product as a template, proceed using step 4b.
- 4a. 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.
- 4b. 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.

5. Mix and centrifuge briefly to collect reactions in the bottom of the tubes.
6. Add 20 μl of Reaction Buffer to the reactions from step 4a or b.
7. Mix and centrifuge briefly to collect reactions in the bottom of the tubes.
8. Add 3.0 μl ($= 0.15 \mu\text{Ci}$) ^{14}C -Leu (300 mCi/mmol) to the reactions from step 7.
9. Mix and centrifuge briefly to collect reactions in the bottom of the tubes.
10. 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.

11. Proceed with sample analysis.

Table 7. 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
<i>E. coli</i> 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	$\sim 0.7 \mu\text{g}$	7 μl	–
EasyXpress Reaction Buffer	20 μl	20 μl	20 μl
^{14}C -Leu (300 mCi/mmol)	3 μl ($=0.15 \mu\text{Ci}$)	3 μl ($=0.15 \mu\text{Ci}$)	3 μl ($=0.15 \mu\text{Ci}$)
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.

Table 8. 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
<i>E. coli</i> 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 (300 mCi/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:

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

Yield (µg/ml)

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

Example

Template:	EasyXpress Positive-Control DNA
Counts:	TCA precipitated radioactivity 1730 cpm/ μ l Total radioactivity 6660 cpm/ μ l
Leu concentration:	1200 μ M
Molecular weight:	31,489 g/mol
Leu residues:	16
% Leu incorporation	$= (1730/6660) \times 100 = 26\%$
Protein yield (μ g/ml)	$= \frac{26 \times 0.01 \times 1200 \mu\text{M} \times 31,489 \text{ g/mol}}{16 \times 1000} = 614 \mu\text{g/ml}$

Appendix D: 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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (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 E: 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 Buffer NP (*Strep*-Tactin Superflow Wash Buffer).**

3. **Store *Strep*-Tactin Superflow resin in Buffer NP (*Strep*-Tactin Superflow Wash Buffer) at 4°C.**

Ordering Information

Product	Contents	Cat. no.
EasyXpress Linear Template Kit Plus (20)	For 20 two-step PCRs: ProofStart DNA Polymerase, buffer, RNase-Free Water, Q-Solution, XE-Solution, positive-control DNA, and optimized PCR primers	32723
EasyXpress pIX3.0 Vector	For protein expression in scaled-up cell-free expression reactions or <i>E. coli</i> : 25 µg vector DNA	32733
EasyXpress Protein Synthesis Kit (5)	For 5 x 50 µl reactions: <i>E. coli</i> extract, reaction buffer, RNase-Free Water, and positive-control DNA	32501
EasyXpress Protein Synthesis Kit (20)	For 20 x 50 µl reactions: <i>E. coli</i> 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 <i>E. coli</i> extract, reaction buffer, RNase-Free Water, and positive-control DNA	32506
EasyXpress Random Biotin Kit	For 60 x 50 µl reactions: biotinyl-lysyl tRNA (Phe)	32612
Related products		
EasyXpress Site-Specific Biotin Kit	For 5 x 25 µl reactions: <i>E. coli</i> 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: <i>E. coli</i> 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: <i>E. coli</i> 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: <i>E. coli</i> extract, amino acid mix w/o X, reaction buffers, RNase-Free Water, gel-filtration columns, and reaction flasks	Varies
EasyXpress Insect Kit II (5)	For 5 x 50 µl reactions: <i>Spodoptera frugiperda</i> insect cell extract, in vitro transcription reaction components, reaction buffers, RNase-Free Water, and positive-control DNA	32561
EasyXpress Insect Kit II (20)	For 20 x 50 µl reactions: <i>Spodoptera frugiperda</i> insect cell extract, in vitro transcription reaction components, reaction buffers, RNase-Free Water, and positive-control DNA	32562
EasyXpress pIX4.0 Vector	25 µg vector DNA for efficient synthesis of proteins using the EasyXpress Insect Kit II	32713
QIAgenes Expression Kit <i>E. coli</i>	QIAgenes Expression Construct <i>E. coli</i> (10 µg), QIAgenes <i>E. coli</i> Positive Control (10 µg), Penta-His Antibody, BSA-free (3 µg), 4 Ni-NTA Spin Columns	Varies
Protein purification		
Ni-NTA Fast Start Kit (6)	For purification and detection of six 6xHis-tagged protein preps: 6 x Fast Start Columns, Penta-His Antibody, Buffers and Reagents	30600
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

* 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.
<i>Strep</i> -Tactin Superflow (2 ml)	For batch and HPLC purification of <i>Strep</i> -tagged proteins: 2 ml <i>Strep</i> -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
Ni-NTA Magnetic Agarose Beads (2 x 1 ml)	2 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36111
<i>Strep</i> -Tactin Magnetic Beads (2 x 1 ml)	For micro-scale purification of <i>Strep</i> -tagged proteins: 2 x 1 ml <i>Strep</i> -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
Protein detection		
Penta-His HRP Conjugate Kit	125 μl Penta-His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer (10x concentrate)	34460
<i>Strep</i> -tag Antibody (100 ug)	Mouse monoclonal antibody that recognizes the <i>Strep</i> -tag II epitope; lyophilized, for 1000 ml working solution	34850
6xHis Protein Ladder	6xHis-tagged marker proteins (lyophilized, for 50–100 lanes on western blots)	34705
Plasmid DNA purification		
HiSpeed Plasmid Midi Kit (25)	25 HiSpeed Midi Tips, 25 QIAfilter Midi Cartridges, 25 QIAprecipitator Midi Modules plus Syringes, Reagents, Buffers	12643

* Not available in all countries, please inquire.

Ordering Information

Product	Contents	Cat. no.
QIAfilter Plasmid Midi Kit (25)	25 QIAGEN-tip 100, Reagents, Buffers, 25 QIAfilter Midi Cartridges	12243
QIAGEN Plasmid Midi Kit (25)	25 QIAGEN-tip 100, Reagents, Buffers	12143
Reverse transcriptases		
Omniscript® RT Kit (50)	For 50 reverse-transcription reactions: 200 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, * RNase-Free Water	205111
Sensiscript® RT Kit (50)	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, * RNase-Free Water	205211

* Contains 5 mM each dNTP.

Notes

Notes

Notes

Trademarks: QIAGEN®, QIAprep®, EasyXpress™, HiSpeed®, LiquiChip®, MinElute®, Omniscript®, Sensiscript® (QIAGEN Group); BIACORE® (Biacore AB); Coomassie® (ICI Organics Inc.); Eppendorf®, Thermomixer® (Eppendorf-Neiheler-Hinz GmbH); Ready Protein+™ (Beckman Coulter, Inc.), *Strep-tag*™, *Strep-Tactin*™ (IBA GmbH); TAGZyme™ (UNIZYME), Tween® (ICI Americas Inc); Whatman® (Whatman PLC); xMAP® (Luminex Corp.)

Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

Strep-tag technology for protein purification and detection is covered by US patent 5,506,121, UK patent 2272698 and French patent 93 13 066; *Strep-Tactin* is covered by US patent 6,103,493.

Hoffmann-La Roche owns patents and patent applications pertaining to the application of Ni-NTA resin (Patent series: RAN 4100/63: USP 4.877.830, USP5.047.513, EP 253 303 B1), and to 6xHis-coding vectors and His-labeled proteins (Patent series: USP 5.284.933, USP 5.130.663, EP 282 042 B1). All purification of recombinant proteins by Ni-NTA chromatography for commercial purposes, and the commercial use of proteins so purified, require a license from Hoffmann-La Roche.

The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. A license under U.S. Patents 4,683,202, 4,683,195, and 4,965,188 or their foreign counterparts, owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd ("Roche"), has an up-front fee component and a running-royalty component. The purchase price of this product includes limited, nontransferable rights under the running-royalty component to use only this amount of the product to practice the Polymerase Chain Reaction ("PCR") and related processes described in said patents solely for the research and development activities of the purchaser when this product is used in conjunction with a thermal cycler whose use is covered by the up-front fee component. Rights to the up-front fee component must be obtained by the end user in order to have a complete license. These rights under the up-front fee component may be purchased from Applied Biosystems or obtained by purchasing an Authorized Thermal Cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR Process may be obtained by contacting the Director of Licensing at Applied Biosystems, 850 Lincoln Center Drive, Foster City, California 94404 or the Licensing Department at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

The 2-step PCR process for generation of linear expression templates developed by RiNA GmbH is patent pending (DE 101 13 265). Generation of an *E.coli* lysate depleted of translation factor Release Factor I (RF1) for use of amber suppressor tRNAs for site-specific labeling is patent pending (DE 10336705.5) The protection of DNA fragments from exonucleolytic digestion developed by RiNA GmbH for use in the EasyXpress system is patented (WO 02/074952).

1051758 © 2005–2008 QIAGEN, all rights reserved.

© 2008 QIAGEN, all rights reserved.

www.qiagen.com

Australia = Orders 03-9840-9800 = Fax 03-9840-9888 = Technical 1-800-243-066

Austria = Orders 0800/28-10-10 = Fax 0800/28-10-19 = Technical 0800/28-10-11

Belgium = Orders 0800-79612 = Fax 0800-79611 = Technical 0800-79556

Canada = Orders 800-572-9613 = Fax 800-713-5951 = Technical 800-DNA-PREP (800-362-7737)

China = Orders 021-51345678 = Fax 021-51342500 = Technical 021-51345678

Denmark = Orders 80-885945 = Fax 80-885944 = Technical 80-885942

Finland = Orders 0800-914416 = Fax 0800-914415 = Technical 0800-914413

France = Orders 01-60-920-926 = Fax 01-60-920-925 = Technical 01-60-920-930 = Offers 01-60-920-928

Germany = Orders 02103-29-12000 = Fax 02103-29-22000 = Technical 02103-29-12400

Hong Kong = Orders 800 933 965 = Fax 800 930 439 = Technical 800 930 425

Ireland = Orders 1800-555-049 = Fax 1800-555-048 = Technical 1800-555-061

Italy = Orders 02-33430411 = Fax 02-33430426 = Technical 800-787980

Japan = Telephone 03-5547-0811 = Fax 03-5547-0818 = Technical 03-5547-0811

Korea (South) = Orders 1544 7145 = Fax 1544 7146 = Technical 1544 7145

Luxembourg = Orders 8002-2076 = Fax 8002-2073 = Technical 8002-2067

The Netherlands = Orders 0800-0229592 = Fax 0800-0229593 = Technical 0800-0229602

Norway = Orders 800-18859 = Fax 800-18817 = Technical 800-18712

Singapore = Orders 65-67775366 = Fax 65-67785177 = Technical 65-67775366

Sweden = Orders 020-790282 = Fax 020-790582 = Technical 020-798328

Switzerland = Orders 055-254-22-11 = Fax 055-254-22-13 = Technical 055-254-22-12

UK = Orders 01293-422-911 = Fax 01293-422-922 = Technical 01293-422-999

USA = Orders 800-426-8157 = Fax 800-718-2056 = Technical 800-DNA-PREP (800-362-7737)

