April 2008

EasyXpress® Site-Specific Biotinylated Protein Synthesis Handbook

For cell-free synthesis of site-specifically biotinylated proteins using *E. coli* lysates

EasyXpress Site-Specific Biotin Kit

QIAGEN®

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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

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Contents

Kit Contents

EasyXpress Site-Specific Biotin Kit For 5 x 25 µl reactions	**Cat. no. 32602**
EasyXpress *E. coli* Extract (SSL)* (red screw-cap)	1 x 40 µl
EasyXpress Reaction Buffer (SSL)* (white screw-cap)	1 x 40 µl
RNase-free Water (colorless screw-cap)	1 x 1.9 ml
EasyXpress Positive-Control DNA (SSL)* (violet screw-cap)	1 x 10 µl
EasyXpress Biotinyl-Lysyl tRNA (amber) (green screw-cap)	1 x 15 µl
Handbook	1

* SSL = Site-Specific Labeling

Storage and Stability

The EasyXpress Site-Specific Biotin Kit is shipped on dry ice. All components must be stored at –70°C. Once thawed, EasyXpress *E. coli* Extract should be stored on ice and used within 4 hours. Do not thaw and refreeze more than four times.

Once thawed, Biotinyl-Lysyl tRNA should be stored on ice and quickly returned to a –70°C freezer after use. Do not thaw and refreeze more than four times.

When stored under the above conditions and handled correctly, the kit can be kept for at least 6 months without showing any reduction in performance.

Product Use Limitations

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

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).

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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

In vitro translation is a widely used tool for the production of recombinant proteins. One of its most important applications is the production of proteins for functional analyses; including activity assays, interaction studies (protein–protein, protein–nucleic acid, and protein–ligand), small molecule inhibition, and the analysis of open reading frames and expression constructs. These studies can be dramatically simplified or even only made possible by labeling protein partners. Adding a specific label or tag at a defined site to different proteins enables their defined immobilization or convenient specific detection. A widely used principle is to tag proteins via biotinylation. With a K_d of 10^{-15} M biotin–(strept)avidin binding is the strongest noncovalent interaction known in nature. This extremely strong binding enables reliable immobilization, purification, and detection strategies, even enabling applications where nonspecific background protein binding requires stringent washing conditions. The biotin moiety greatly facilitates detection and immobilization of recombinant proteins in BIACORE®, ELISA, xMAP®, protein microarrays, and other assay applications.

QIAGEN offers the EasyXpress Site-Specific Biotin Kit for site-specific cotranslational biotinylation of proteins. Using in vitro translation in *E. coli* extracts, recombinant biotinylated proteins are produced in one step from DNA templates encoding the biotin moiety at a single codon. There is no need for posttranslational enzymatic biotinylation of larger recognition sequences that may affect protein structure and activity, or posttranslational chemical treatment with biotin containing chemicals, which leads to random biotinylation of proteins and in many cases, the subsequent inactivation of their biological activities.

The EasyXpress Site-Specific Biotin Kit directs the incorporation of a biotin labeled amino acid at any desired position in the amino acid sequence of a recombinant protein. This allows optimization of the biotin tag position, for example, to expose a certain protein epitope to interaction partners or detection reagents. The small size of the biotin moiety — which is added to the protein as a single biotinylated amino acid — minimizes changes of the protein structure often associated with the addition of larger peptide tags, providing optimal activity of the biotin-tagged protein.

Principle and Procedure

The unique EasyXpress Site-Specific Biotin Kit (patent pending) enables insertion of a biotin label at a defined position in the amino acid sequence of a recombinant protein. This insertion is accomplished using a synthetic aminoacylated tRNA that carries a lysine residue labeled at the epsilon position with biotin and an anticodon that recognizes the UAG amber stop codon (Figure 1).

Recombinant proteins are synthesized by highly productive EasyXpress *E. coli* lysates that are treated to reduce the levels of release factor 1 (RF1), a protein whose presence would cause translation termination at the amber codon. This enables translation to continue through the amber codon and efficient incorporation of the biotinylated residue in the growing protein chain (reference 1). Site-specific biotin labeling enables a single biotin moiety to be introduced at any desired position within a protein's amino acid sequence (except the start methionine). This allows selection of a label position which does not interfere with protein structure and function, greatly facilitating protein assays. Incorporation of biotin occurs at a stoichiometry of 1:1, i.e., one biotin molecule per protein molecule (see Figure 2).

The kit directs the incorporation of the biotin tag at the UAG (amber) codon. In the DNA expression template, the coding triplet corresponding to the selected biotin position is replaced by the amber stop codon (TAG). This can be achieved using the EasyXpress Linear Template Kit Plus and customer-designed primers which contain the amber codon, or by using any site-directed in vitro mutagenesis method.

The EasyXpress Site-Specific Biotin Kit uses highly productive *E. coli* cell lysates, which contain all translational machinery components (ribosomes, ribosomal factors, tRNAs, aminoacyl-tRNA synthetases, etc.) as well as T7 RNA polymerase. It uses a coupled transcription–translation system that can be used to express full-length proteins from T7 or *E. coli* T5 promoters in a single-step reaction using plasmid or linear DNA templates, such as PCR products. The *E. coli* lysate used for in vitro translation with the EasyXpress Site-Specific Biotin Kit has been adapted to the specific requirements of site-specific labeling. The lysate possesses very fast synthesis kinetics and the levels of some translation factors important for efficient synthesis of site-specifically labeled proteins have been modified. Endogenous biotinylated proteins normally present in prokaryotic lysates have been removed, greatly facilitating assays that make use of the biotin moiety for protein immobilization and detection (see Figure 3). For many of these applications it is not necessary to purify the biotinylated proteins.

Using the EasyXpress Site-Specific Biotin Kit, up to 5 µM (≈150 µg/ml) biotin-labeled protein can be synthesized within 30 minutes. The synthesis reaction can be easily scaled up or down (for high-throughput screening in 96-well plate format). The amount of protein synthesized increases linearly with reaction volume.

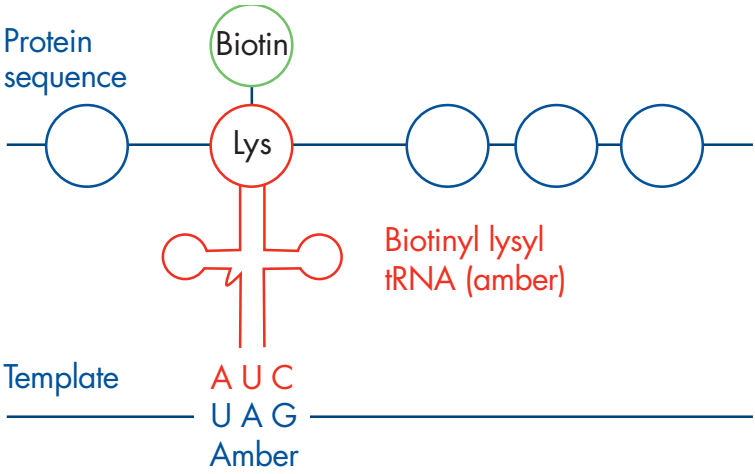


Figure 1. Schematic representation of biotin incorporation using the EasyXpress Site-Specific Biotin Kit.

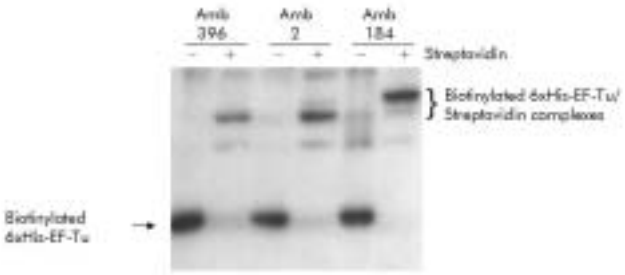


Figure 2. Native gel electrophoresis demonstrates 1:1 stoichiometry of biotin incorporation. Three C-terminally His-tagged variants of the *E. coli* EF-Tu gene containing an amber codon at positions 2 (**Amb 2**), 184 (**Amb 184**), or 396 (**Amb 396**) respectively, were synthesized using the EasyXpress Site-Specific Biotin Kit. Following protein synthesis, reaction aliquots were incubated in the presence or absence of streptavidin, subjected to native gel electrophoresis, and visualized using a Penta·His HRP Conjugate after Western Blotting. The electrophoretic mobility of the biotinylated EF-Tu/streptavidin complexes is less than that of free EF-Tu and as a result the EF-Tu (which is visualized via its His tag) appears to be shifted to a higher molecular weight. Irrespective of the amber codon position, EF-Tu was almost completely shifted by streptavidin, indicating a stoichiometric biotin incorporation (i.e., each protein molecule contains one biotin moiety).

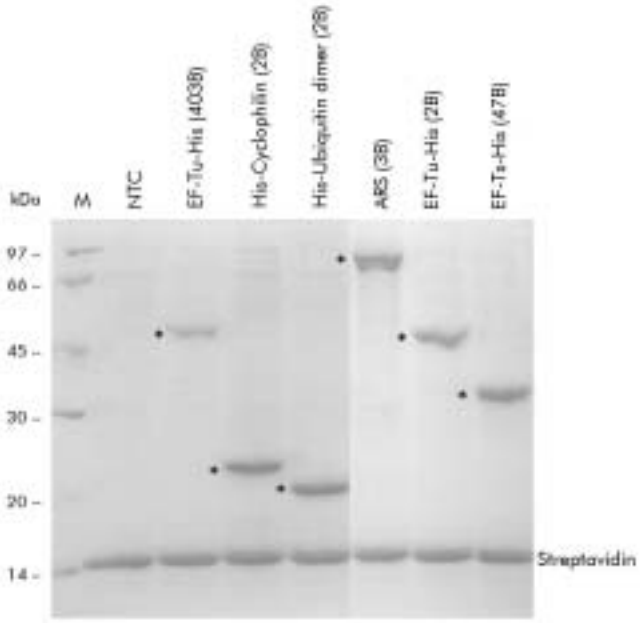


Figure 3. Efficient immobilization of site-specifically biotinylated proteins direct from synthesis reactions. Various biotinylated proteins were synthesized in 25 µl reactions using the EasyXpress Site-Specific Biotin Kit and immobilized on *Strep*-Tactin™ magnetic beads (QIAGEN, cat. no. 36311). Beads were washed once with PBS to remove nonspecifically bound proteins. Washed beads were boiled in sample buffer and supernatants separated by SDS-PAGE. Proteins were visualized by Coomassie staining. Immobilized site-specifically biotinylated proteins are indicated by asterisks. **M**: markers. **NTC**: no template control.

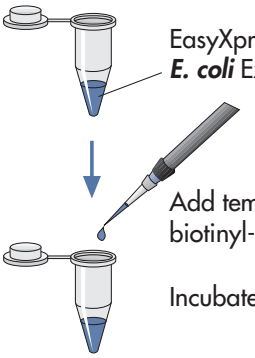
EasyXpress Site-Specific Biotinylated Protein Synthesis Procedure

Generate expression template

EasyXpress Linear Template Kit Plus

EasyXpress pIX3.0 Vector

QIAgenes Expression Construct



EasyXpress Site-Specific Biotin Kit

Optional: purify for downstream applications (His-tagged proteins)

Ni-NTA matrices, e.g., Ni-NTA Magnetic Agarose Beads or Ni-NTA Superflow Cartridge

Immobilize proteins directly for downstream applications, e.g., protein interaction studies

Strep-Tactin Magnetic Beads

BioMag Streptavidin

Streptavidin Magnetic Beads

Protein detection/assay

Streptavidin R-PE

Streptavidin POD Conjugate (Roche)

Penta·His Antibody or HRP Conjugate (His-tagged 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 4).

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 gene10 have been reported to enormously improve protein synthesis (3). Suitable DNA templates include linear DNA (e.g. PCR products) and supercoiled plasmids.

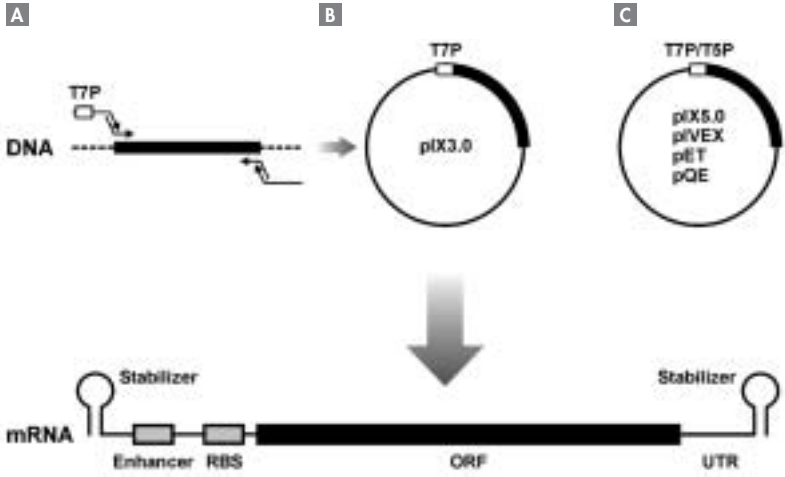


Figure 4. Different types of DNA templates compatible with the EasyXpress System (top) and optimal organization of an mRNA for efficient in vitro translation (bottom). A Linear template generated with the EasyXpress Linear Template Kit Plus. B Linear template subcloned into vector pIX3.0. C Other vectors 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 4B).

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 Vectors

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

Generating DNA Templates for Co-translational Site-Specific Biotinylation

Choosing the best position for biotin placement

The principle of the EasyXpress Site-Specific Biotin Kit is to incorporate the biotin tag as a single biotinylated amino acid (biotinylated lysine, biocytin) encoded within the protein gene by a single codon (amber, UAG). This allows placing the biotin at any desired position within the protein by changing a single amino acid side chain. It is recommended that the biotin moiety is incorporated at a freely accessible site within the protein. Since accessible sites are often near the N- or C-terminus of the protein, as an initial approach we recommend regarding the biotin tag as a peptide tag and placing the biotin within these regions.

Accessible sites providing optimal signal strength and maintaining maximal protein activity can often be found on the protein surface remote from the N- or C-termini. If available, refer to the literature on the protein of interest (e.g. 3-D structure, hydrophobicity plots, and known silent mutations) to find a suitable position for internal biotin placement. Hydrophobic stretches are not usually exposed to the surface of the protein and therefore may not be accessible to detection molecules or immobilization matrices. If no information is available it is recommended that several positions are evaluated in parallel.

It is possible to use the same template for expression of both labeled and unlabeled protein if the amber stop codon (UAG) is inserted directly before the termination codons UAA or UGA. Using the native stop codon context of the C-terminus for biotin placement may result in reduced biotin incorporation efficiency. Biotin incorporation at the C-terminus may be improved using the codon sequence UAG AAA UAA, i.e., Amber – Lys – Stop.

Creating an amber codon for site-directed incorporation of a biotin moiety

For site-specific incorporation of a biotin moiety, the codon of the amino acid residue that will be replaced by biotinylated lysine is replaced by an amber stop codon (UAG) at the respective position within the DNA template. A convenient strategy for incorporation of a biotin moiety near the N- or C-terminus of a protein of interest is to introduce the amber codon via one of the gene-specific primers used to generate an expression template with the EasyXpress Linear Template Kit Plus. The procedure is described in detail below.

Alternatively, introduction of a mutation at any desired position within the coding region can be achieved by 2-step mutation PCR or by site-directed in vitro mutagenesis (see Appendix A, page 38 for protocol).

Important: To ensure correct synthesis of the C-terminal end of the protein, ensure that the coding sequence of the protein gene ends with one of the termination codons UAA or UGA, which are both recognized by the translation machinery of the EasyXpress *E. coli* extract as translation stop signals. If using the EasyXpress Linear Template Kit Plus for template generation, the termination codon will be UAA.

Generating DNA Templates for Co-translational Site-Specific Biotinylation Using the EasyXpress Linear Template Kit Plus

The EasyXpress Linear Template Kit Plus uses a two-step procedure to generate PCR products suitable for in vitro translation in EasyXpress *E. coli*- and insect-cell–based kits. In the first step, defined 5'-tails are added to PCR products using gene-specific primers that are designed and supplied by the user. The 5'-tails serve as hybridization sites for primers used in a second PCR, in which DNA is amplified using adapter primers that code for regulatory elements required for optimal expression. These elements include a T7 promoter, ribosomal binding site, and T7 terminator (see Figure 4, page 12). Different adapter primers enabling addition of N- or C-terminal affinity tags are contained in the kit. The combination of ProofStart® DNA Polymerase — a unique hot-start high-fidelity enzyme — and ProofStart PCR Buffer (both supplied in the kit) minimizes the need for PCR optimization and optimizes PCR product yield.

Plasmid DNA, genomic DNA mixtures, or cDNA mixtures can be used as a template for the first PCR. Alternatively, cDNA can be generated by reverse transcription PCR (RT-PCR) using a gene-specific antisense primer, total RNA, and a reverse transcriptase. QIAGEN offers Omniscript® and Sensiscript® RT Kits for efficient RT-PCR.

The final PCR product can be added to the in vitro translation reaction without any further purification steps.

Rules for the design of the gene-specific PCR primers containing the relevant 5' tails are given in the following chapter. For further information on the EasyXpress Linear Template Kit Plus please refer to the *EasyXpress Linear Template Kit Plus Handbook*.

Strategy for designing gene-specific primers

Prerequisites for successful PCR include the design of optimal gene-specific primer pairs, the use of appropriate primer concentrations, and the correct storage of primer solutions. For best results, primers should be HPLC-purified.

The final PCR product added to the in vitro translation reaction is generated by a two-step PCR procedure. In the first PCR, primers must be designed that are not only specific for the protein of interest, but also provide 5' tails that will act as hybridization sites for adapter primers used in the second PCR. Because the biotin tag is suitable for immobilization, detection, and purification the first step in designing primers is to decide whether an additional affinity tag is required or not. For applications where an additional affinity tag is required, e.g., as an alternative protein purification tool or as additional detection epitope, the combination of a biotin residue near the N-terminus with a C-terminal affinity tag is most suitable. Alternatively, an N-terminal tag may be used in combination with a C-terminal (or internal) biotin residue.

Use the sequence information in Tables 1 and 2 and the information below to design forward (sense) and reverse (antisense) primers for protein constructs with (or without) an affinity tag at the respective terminus. For further information on immobilization or purification of biotinylated proteins produced using the EasyXpress Site-Specific Biotin Kit please refer to the sections “Immobilization of Biotinylated Proteins” (page 20) and “Purification of Biotinylated Proteins” (page 20), respectively.

For incorporation of a biotin moiety near the N- or C-terminus of the protein, the UAG (amber) codon can be easily introduced via one of the gene-specific primers. Table 1 gives examples of gene-specific primers that enable incorporation of a biotin moiety as the second or penultimate amino acid, including optional combinations with a His tag. In these examples the UAG (amber) codon is added to the ORF as an additional codon. Other biotin positions are possible by introducing amber mutations into the gene-specific primer sequence at different positions. If using the EasyXpress Linear Template Kit Plus for the generation of PCR expression templates from DNA that already contains an (internal) amber codon for biotin placement, use the sequence information in Table 2.

Note: Theoretically, biotin can also be incorporated in combination with a *Strep*-tag™II. However, the presence of the biotin residue prevents using the *Strep*-tag for affinity purification on *Strep*-Tactin matrices. However the presence of the biotin moiety does not prevent using the *Strep*-tag as a recognition epitope for the *Strep*-tag Antibody (cat. no. 34850) in assays in which the biotinylated residue is used for protein immobilization.

Length

- The length of the gene-specific sequence should be 17–20 bases (see Table 2). This may be reduced or increased in some cases to give primers suitable for the annealing temperature of 50°C.

Melting temperature (T_m)

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

Simplified formula for estimating melting temperature (T_m):

T_m = 2°C x (A+T) + 4°C x (G+C)

Whenever possible, design primer pairs with similar T_m values. If introducing amber mutations via the gene-specific primers ensure that the annealing temperature of the sequence 3' of the amber codon is 50°C.

Table 1. Example sequences of gene-specific primers (required for first-round PCR) used for site-specific incorporation of a biotinylated residue near the N- or C-terminus of a protein

Affinity tag	Position of biotin	Forward sense primer sequence	Reverse antisense primer sequence
No tag	Amino acid 2	5'-AGA AGG AGA TAA ACA **ATG** *TAG* N_{17}-3' Met Biotin	5'C TTG GTT AGT TAG TTA **TTA** X_{20}-3' Stop
C-terminal 6xHis tag	Amino acid 2	5'-AGA AGG AGA TAA ACA **ATG** *TAG* N_{17}-3' Met Biotin	5'-TG *GTG* ATG GTG GTG ACC CCA X_{20}-3' His His His Gly Trp
No tag	Penultimate amino acid	5'-AGA AGG AGA TAA ACA **ATG** N_{17}-3' Met	5'-C TTG GTT AGT TAG TTA **TTA** TTT *CTA* X_{20}-3' Stop Lys Biotin
N-terminal 6xHis tag	Penultimate amino acid	5'-AT CAC CAT CAC CAC GGT ATG N_{17}-3' His His His His Gly Met	5'-C TTG GTT AGT TAG TTA **TTA** TTT *CTA* X_{20}-3' Stop Lys Biotin

N_{17} represents the first 17 sense-strand bases of protein-encoding sequence to which the primers will hybridize. To amplify sequence that does not contain an ATG start codon, extend the 3' end of the primer by 3 bases.

X_{20} represents the first 20 antisense-strand bases of protein-encoding sequence to which the primers will hybridize.

The amber biotin stop codon is in italics (TAG; reverse CTA)

Shaded sequence is an absolute requirement for hybridization of adapter primers used in the second PCR, and must form the 5'-terminal sequence of the gene-specific primers supplied by the user.

Translation initiation codons (ATG) and stop codons (TAA) are in bold. Underlined codons replace endogenous stop codons. Upstream codons must be cloned in frame.

To enhance incorporation efficiency of a biotin moiety as the last amino acid of the protein, an additional codon AAA (Lys) is placed between the amber codon (TAG; reverse CTA) and the stop codon (TAA).

Table 2. Gene-specific primer sequences (required for first-round PCR) used to generate expression PCR products from DNA templates containing an internal amber codon for biotin placement

Affinity tag	Forward sense primer sequence	Reverse antisense primer sequence
No tag	5'-AGA AGG AGA TAA ACA **ATG** N_{17}-3' Met	5'C TTG GTT AGT TAG TTA **TTA** X_{20}-3' Stop
C-terminal 6xHis tag	5'-AGA AGG AGA TAA ACA **ATG** N_{17}-3' Met	5'-TG GTG ATG GTG GTG ACC CCA X_{20}-3' His His His His Gly Trp
N-terminal 6xHis tag	5'-AT CAC CAT CAC CAC GGT ATG N_{17}-3' His His His His Gly Met	5'-C TTG GTT AGT TAG TTA **TTA** X_{20}-3' Stop

N_{17} represents the first 17 sense-strand bases of protein-encoding sequence to which the primers will hybridize. To amplify sequence that does not contain an ATG start codon, extend the 3' end of the primer by 3 bases.

X_{20} represents the first 20 antisense-strand bases of protein-encoding sequence to which the primers will hybridize.

Shaded sequence is an absolute requirement for hybridization of adapter primers used in the second PCR, and must form the 5'-terminal sequence of the gene-specific primers supplied by the user.

Translation initiation codons (ATG) and stop codons (TAA) are in bold. Underlined codons replace endogenous stop codons. Upstream codons must be cloned in frame.

Immobilization of Biotinylated Proteins

For many applications (e.g., constructing protein microarrays, or BIACORE and xMAP studies) proteins must be immobilized on a solid support. With a K_d of 10^{-15} M, avidin/streptavidin-biotin binding is the strongest noncovalent interaction known in nature. Consequently, the biotin tag is well-suited for efficient immobilization of proteins on different surfaces and allows stringent washing conditions. The avidin/streptavidin–biotin complex is compatible with a broad range of buffer conditions — even denaturing conditions (e.g., up to 1% SDS, 3 M guanidine hydrochloride or 6 M urea) — enabling immobilization conditions to be adapted to the individual requirements of the respective protein. *Strep*-Tactin-coated surfaces or beads (5) are equally suitable for binding of proteins and other biomolecules carrying biotin moieties.

Since the *E. coli* lysates used in the EasyXpress Site-Specific Biotin Kit are pre-treated to remove endogenous biotinylated proteins, biotinylated proteins can usually be immobilized directly from the EasyXpress Site-Specific Biotin Kit reactions without prior purification, avoiding additional processing steps. This allows immobilization of proteins that are difficult to purify. In addition, there is no need for removal of free biotin prior to immobilization, since precharged biotinylated amber suppressor tRNA (EasyXpress Biotinyl-Lysyl tRNA (amber)) is the only source of biotin, and will mostly be incorporated into protein. The negligible amount of free biotin arising from spontaneous hydrolysis of the amino acyl bond at the tRNA does not normally affect downstream applications. The concentration of biotinylated tRNA in the reaction is 12 µM, which corresponds to 300 pmol per 25 µl translation reaction. Therefore, the amount of matrix (Avidin, Streptavidin, *Strep*-Tactin, etc.) added to the completed protein synthesis reactions should have a minimum binding capacity of 300 pmol biotin per 25 µl reaction to enable immobilization of the total amount of biotin within the system (see protocol in Appendix B, page 41).

Purification of Biotinylated Proteins

Because endogenous biotinylated proteins are removed from the *E. coli* lysates used in the EasyXpress Site-Specific Biotin Kit, purification of proteins before immobilization is not usually necessary. Detection of biotin-labeled protein by western blot analysis does not require prior purification.

However, there may be applications where the protein background from the *E. coli* lysate or non-biotinylated truncated translation products may interfere with downstream assays. Purification of recombinant biotinylated proteins is also advisable if proteins are known to easily fragment. To avoid the purification of truncated translation products, and to ensure that only full-length protein is purified, proteins should be purified using C-terminal tags.

Due to the extraordinary stability of the streptavidin-biotin complex, if you wish to use the biotin moiety for purification, special lower affinity matrices (e.g., streptavidin mutein) should be used, since the release of biotinylated proteins from streptavidin (and avidin and *Strep*-Tactin) is not possible using mild non-denaturing conditions. For the same reason, it is not advisable to purify biotinylated in vitro translated proteins using a *Strep*-tag, as the biotin moiety will bind with extremely high affinity to *Strep*-Tactin matrices, effectively immobilizing the protein. However, the *Strep*-tag epitope can still be used for immunolocalization using the *Strep*-tag Antibody. Note that the antibody must be added to the system before the biotin–(strept)avidin interaction occurs, because otherwise the *Strep*-tag may be masked by biotin binding to (strept)avidin.

Purification of tagged proteins using the 6xHis-tag–Ni-NTA interaction

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

NTA, which has four chelation sites for nickel ions, binds nickel more tightly than metal-chelating purification systems that only have three sites available for interaction with metal ions. The extra chelation site prevents nickel-ion leaching; providing a greater binding capacity, and high-purity protein preparations. For purification protocols, see Appendix C, page 44.

Protocol: Two-Step PCR Procedure for Generating an Expression Template Using the EasyXpress Linear Template Kit Plus

This protocol is made up of two separate PCR procedures. In the first PCR, protein-specific sequence is used as a template. The primers used in this first PCR add sequences that will serve as hybridization sites in a second round of PCR (see the *EasyXpress Linear Template Kit Handbook*). In the second PCR, adapter primers are used to add sequences that encode affinity tags and/or regulatory elements required for efficient expression.

Important points before starting

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

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

Procedure: First PCR using gene-specific primers

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

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

Table 3. PCR components (master mix and template DNA)

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

* contains 15 mM $MgSO_4$

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Procedure: Second PCR using adapter primers

9. **Thaw 10x ProofStart PCR Buffer (blue screw-cap), dNTP mix (purple screw-cap), primer solutions, and, if required, 25 mM $MgSO_4$ (yellow screw-cap). Mix the solutions thoroughly before use.**

10. **Prepare a master mix according to Table 4.**

Table 4. PCR components (master mix and template DNA)

Component	Volume/reaction	Final concentration
Master mix		
10x ProofStart PCR Buffer*	2.5 µl	1x
dNTP (10 mM of each)	0.75 µl	300 µM of each dNTP
Sense Adapter Primer†	2 µl†	
Antisense Adapter Primer†	2 µl†	
ProofStart DNA Polymerase	● 0.5 µl‡; ▲ 1 µl‡	● 1.25 units; ▲ 2.5 units
RNase-Free Water	variable	—
Template DNA		
Template DNA, added in step 12	1–2 µl product from first PCR (~100 ng)	
Total volume	**25 µl**	—

* Contains 15 mM $MgSO_4$

† Adapter primers are supplied in the EasyXpress Linear Template Kit Plus.

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

11. **Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes.**

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

12. **Add template DNA (1–2 µl of the first PCR, ~100 ng) to the individual tubes containing the master mix.**

13. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 14. Otherwise, overlay with approximately 25 µl mineral oil.**

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

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

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

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

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

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

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

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

    0.35 µg DNA (~3.5 µl of the second PCR) is required for a 25 µl in vitro translation reaction.

Protocol: In Vitro Translation with Site-Specific Biotinylation Using a PCR Product as a Template

This protocol is suitable for site-specific co-translational biotinylation of recombinant proteins during in vitro translation from PCR products using the EasyXpress Site-Specific Biotin Kit. If PCR products are added to in vitro translation reactions, we recommend that they are generated using the EasyXpress Linear Template Kit Plus. PCR products can be added directly to in vitro translation reactions without further cleanup.

Reactions can be easily scaled down to as little as 5 µl for high-throughput screening in 96-well microplate format. The reaction volumes given in Table 5 should be adjusted accordingly. We recommend using flat-bottom polystyrene 96-well plates (e.g., Greiner Bio-One, cat. no. 655101) for reaction volumes >25 µl and 96-well thin-wall polycarbonate PCR plates (e.g., Corning Incorporated, cat. no. 6513) for reaction volumes <25 µl. To avoid evaporation, microplates and PCR plates should be sealed with a tape sheet during the in vitro translation reaction in an incubator or on a PCR cycler.

Equipment and reagents to be supplied by user

- PCR template encoding the protein of interest which has an amber stop codon at the desired position of biotin incorporation (see protocol on page 22)
- Thermomixer® (Eppendorf, Hamburg, Germany), water-bath, or heating block

Important points before starting

- The in vitro translation system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Do not add Biotinyl-Lysyl-tRNA to a master mix. This may result in lower amounts of biotin incorporation due to deacylation of the tRNA carrying the biotinylated residue. Start the reaction by adding Biotinyl-Lysyl-tRNA to the master mix as recommended in the protocol.
- For multiple reactions, prepare a master mix without template and Biotinyl -Lysyl-tRNA, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the Biotinyl-Lysyl-tRNA and the template.
- Biotinyl-Lysyl tRNA and *E. coli* extract are very sensitive to multiple freeze–thaw cycles. Once thawed, keep on ice, and use within 4 hours. Replace in a freezer at –70°C immediately after use. Do not thaw and refreeze more than four times.
- Except for the actual transcription-translation incubation, all handling steps should be carried out on ice.

- To determine the background level of protein synthesis, include a no template control reaction (without template) in your experiment.
- The recommended incubation temperature for protein synthesis is 37°C, but lower incubation temperatures may improve protein solubility in some cases.
- The functionality of the kit can be checked by performing a positive control reaction containing EasyXpress Positive-Control DNA (SSL) that encodes the 32 kDa elongation factor EF-Ts with a C-terminal 6xHis tag and an amber stop mutation at amino acid position 47.

Procedure

1. **Thaw EasyXpress Reaction Buffer (SSL) (white screw-cap), EasyXpress *E. coli* Extract (SSL) (red screw-cap), EasyXpress Biotinyl-Lysyl tRNA (amber) (green screw-cap) and XE-Solution (green screw-cap) on ice. Thaw RNase-free Water (colorless screw-cap) at room temperature (15–25°C).**

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

2. **Calculate the amount of RNase-free Water needed to bring the final reaction volume to 25 µl. Pipet the calculated amount of RNase-free Water, 8 µl EasyXpress Reaction Buffer (SSL), and 8 µl EasyXpress *E. coli* Extract (SSL) into a 1.5 ml microcentrifuge tube.**

   Table 5 provides a pipetting scheme for EasyXpress site-specific biotin labeling reactions using PCR products.

3. **For each reaction add 1 µl XE-Solution to 0.35 µg DNA (~3.5 µl) from the second PCR in a separate tube 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 or the Biotinyl-Lysyl tRNA (amber). For the no template control reaction, mix 1 µl XE-Solution and 3.5 µl RNase-free water.

4. **Add the ~4.5 µl XE-Solution-PCR product mix from step 3 to each reaction.**

5. **Add 3 µl EasyXpress Biotinyl-Lysyl tRNA (amber) to each reaction.**

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

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

7. **Incubate the reactions at 37°C for 30 min.**

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

8. **Stop the reaction by placing on ice and proceed with sample analysis.**

   The 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 1–3 µl of the in vitro translation reaction is sufficient for one gel lane.

Table 5. Pipetting scheme for setup of EasyXpress site-specific biotin labeling reactions using a PCR product as a template

Component	In vitro translation samples	No template control
RNase-free Water	Add to 25 µl	5 µl
EasyXpress Reaction Buffer (SSL)	8 µl	8 µl
EasyXpress *E. coli* Extract (SSL)	8 µl	8 µl
XE-Solution*	1 µl	1 µl
DNA template from second PCR	0.35 µg	–
EasyXpress Biotinyl-Lysyl-tRNA (amber)	3 µl	3 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 (SSL).

Protocol: In Vitro Translation with Site-Specific Biotinylation Using Plasmid DNA as a Template

This protocol is suitable for site-specific co-translational labeling of recombinant proteins during in vitro translation from plasmid DNA using the EasyXpress Site-Specific Biotin Kit.

Reactions can be easily scaled down to as little as 5 µl for high-throughput screening in 96-well microplate format. The reaction volumes given in Table 6 should be adjusted accordingly. We recommend using flat-bottom polystyrene 96-well plates (e.g., Greiner Bio-One, cat. no. 655101) for reaction volumes >25 µl and 96-well thin-wall polycarbonate PCR plates (e.g., Corning Incorporated, cat. no. 6513) for reaction volumes <25 µl. To avoid evaporation, microplates and PCR plates should be sealed with a tape sheet during the in vitro translation reaction in an incubator or on a PCR cycler.

Equipment and reagents to be supplied by user

- Plasmid DNA template encoding the protein of interest with an amber stop codon (UAG) at the desired position of biotin incorporation
- Thermomixer (Eppendorf, Hamburg, Germany), water-bath, or heating block

Important points before starting

- The in vitro translation system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Do not add Biotinyl-Lysyl-tRNA to a master mix. This may result in lower amounts of biotin incorporation due to deacylation of the tRNA carrying the biotinylated residue. Start the reaction by adding Biotinyl-Lysyl-tRNA to the master mix as recommended in the protocol.
- For multiple reactions, prepare a master mix without template and Biotinyl-Lysyl-tRNA, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the Biotinyl-Lysyl-tRNA and the template.
- Biotinyl-Lysyl tRNA and *E. coli* extract are very sensitive to multiple freeze–thaw cycles. Once thawed, keep on ice, and use within 4 hours. Replace in a freezer at –70°C immediately after use. Do not thaw and refreeze more than four times.
- Except for the actual transcription-translation incubation, all handling steps should be carried out on ice.
- To determine the background level of protein synthesis, include a no template control reaction (without template) in your experiment.

- The recommended incubation temperature for protein synthesis is 37°C, but lower incubation temperatures may improve protein solubility in some cases.
- The functionality of the kit can be checked by performing a positive control reaction containing EasyXpress Positive-Control DNA (SSL) that encodes the 32 kDa elongation factor EF-Ts with a C-terminal 6xHis tag and an amber stop mutation at amino acid position 47.
- If plasmid DNA template contains IPTG inducible promoter systems (e.g., T5 or T7) add IPTG (final concentration 1mM) to the translation reaction.

Procedure

1. **Thaw EasyXpress Reaction Buffer (SSL) (white screw-cap), EasyXpress *E. coli* Extract (SSL) (red screw-cap) and EasyXpress Biotinyl-Lysyl tRNA (amber) (green screw-cap) on ice. Thaw RNase-free Water (colorless screw-cap) and EasyXpress Positive-Control DNA (SSL) (violet screw-cap) at room temperature (15–25°C).**

   Gently vortex all components before use.

2. **Calculate the amount of RNase-free Water needed to bring the final reaction volume to 25 µl. Pipet the calculated amount of RNase-free Water, 8 µl EasyXpress Reaction Buffer (SSL), and 8 µl EasyXpress *E. coli* Extract (SSL) into a 1.5 ml microcentrifuge tube.**

   Table 6 provides a pipetting scheme for EasyXpress site-specific biotin labeling reactions using plasmid DNA as template.

3. **Add 0.25–0.5 µg plasmid DNA to each reaction tube. Add 2.5 µl EasyXpress Positive-Control DNA (SSL) to the positive-control reaction tube. Do not add any DNA to the no template control reaction tube.**

   The amount of plasmid DNA added to each 25 µl in vitro translation reaction should be 0.25 µg for plasmids up to 5 kb in size or 0.5 µg for plasmids >5 kb.

4. **Add 3 µl EasyXpress Biotinyl-Lysyl tRNA (amber) to each reaction.**

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

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

6. **Incubate the reactions at 37°C for 30 min.**

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

7. **Stop the reaction by placing on ice and proceed with sample analysis.**

   Biotin labelled proteins can be detected after separation on SDS-PAGE and subsequent blotting by colorimetric or chemiluminescent visualization with Streptavidin-AP or Streptavidin-HRP. Usually 1–3 µl of the in vitro translation reaction is sufficient for one gel lane.

Table 6. Pipetting scheme for setup of EasyXpress site-specific biotin labeling reactions using plasmid DNA as a template

Component	In vitro translation samples	No template control	Positive control
RNase-free Water	Add to 25 µl	6 µl	3.5 µl
EasyXpress Reaction Buffer (SSL)	8 µl	8 µl	8 µl
EasyXpress *E. coli* Extract (SSL)	8 µl	8 µl	8 µl
Plasmid DNA template	0.25 µg	–	2.5 µl*
EasyXpress Biotinyl-Lysyl-tRNA (amber)	3 µl	3 µl	3 µl

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

Troubleshooting Guide

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

		Comments and suggestions
First and second PCR		
Please refer to the Troubleshooting Guide in the handbook supplied with the EasyXpress Linear Template Kit Plus.		
In vitro translation		
No control protein visible		
a)	Reaction was contaminated with RNases	Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
b)	Kit has not been stored at –70°C	The *E. coli* extracts in EasyXpress Kits will remain fully active for just 24 h if stored at temperatures of –20°C or above. After this time the extracts will lose activity.
c)	Pipetting error or missing reagent	Check the pipets used for experimental setup. Mix all reagents well after thawing, and repeat in vitro translation.
d)	Incorrect setup temperature	Be sure to set up the reaction on ice.
No target protein but normal expression of control protein		
a)	Poor quality or wrong quantity of DNA template	Check the concentration, integrity, and purity of the DNA template. Prepare high-purity plasmid DNA with QIAGEN plasmid kits and optimized linear expression templates with the QIAGEN EasyXpress Linear Template Kit. Determine the optimal amount of DNA template used in the in vitro translation by titration.

		Comments and suggestions
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 check the strategy for designing gene-specific primers.
c)	In vitro transcription or in vitro translation is disrupted by expressed protein	Express control protein in the presence of the target protein. If expression of control protein is inhibited, it may not be possible to express the target protein using the EasyXpress Protein Synthesis System.
d)	Rigid secondary structures in the mRNA inhibit initiation of translation	Include a 6xHis-tag coding sequence at the 5' end of the protein coding sequence. If the protein to be expressed already contains a tag, move the tag to the opposite terminus.
Low expression yield		
a)	Contamination with RNases	Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
b)	Poor quality or wrong quantity of DNA template	Check the concentration, integrity, and purity of the DNA template. Prepare high-purity plasmid DNA with QIAGEN plasmid kits and optimized linear expression templates with the QIAGEN EasyXpress Linear Template Kit Plus. Determine the optimal amount of DNA template used in the in vitro translation by titration.
c)	If protein is expressed with a tag, the tag has a negative effect on expression of the protein	Introduce different tag sequences at different positions (N-terminal or C-terminal) via PCR using the EasyXpress Linear Template Kit. This can significantly affect protein expression and solubility (4).
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.

		Comments and suggestions
e)	Plasmid DNA template contains *E. coli* promoter (e.g., T5 or lac)	Add IPTG (final concentration 1 mM) to translation reaction to overcome endogenous lac repressor.
Sufficient protein expression, but low yield of active protein		
a)	Incorrect folding of the protein due to dependence on posttranslational modifications	*E. coli* lysate cannot introduce posttranslational modifications like glycosylation, phosphorylation, or signal-peptide cleavage.
b)	Cofactors required for activity	Add cofactors to synthesis reaction and/or activity assay (see Appendix D, page 48).
Expressed protein is insoluble		
Protein forms aggregates		We recommended a 37°C incubation temperature for protein synthesis, but lower incubation temperatures may improve protein solubility.
Expressed protein is degraded		
a)	Premature termination of translation	Check reading frame of the target sequence for mutations that might produce a stop codon.
b)	Protein is degraded by proteases	Add protease inhibitors to the in vitro translation reaction.
No expression of biotinylated protein/no biotin incorporation		
Template does not contain an amber codon or codon is out of frame		Ensure that the template contains an in frame amber codon (UAG) at the desired position of biotin incorporation within the coding sequence of the protein. Check for biotin incorporation by performing a control reaction using the control plasmid and Biotinyl-Lysyl-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.

	Comments and suggestions
Full-length protein expressed but biotin not incorporated	
No tRNA added to reaction	Sometimes unlabelled full-length products are observed due to read through in the absence of RF1. This read-through effect is suppressed in the presence of Biotinyl-Lysysl-tRNA.
Both labeled and unlabeled full-length protein expressed	
Reaction time exceeded 30 min	During the 30 min reaction time the Biotinyl-Lysyl tRNA is used up and read through may occur if the reaction time is extended. Reaction time should be 30 min as recommended in the protocol.
Poor incorporation of biotin/weak signal on western blot or in interaction assay	
a) Biotinyl-Lysyl-tRNA added to master mix	Adding Biotinyl-Lysyl tRNA to a master mix may result in lower amounts of biotin incorporation due to deacylation of the tRNA carrying the biotinylated residue. Work quickly and start the reaction by adding Biotinyl-Lysyl-tRNA as recommended in the protocol.
b) Detection reagents are not functioning correctly	Perform a control reaction to ensure that all compounds used for detection of the biotin moiety are functioning correctly.
c) Biologically active, non-biotinylated, truncated translation products reduce the sensitivity of the assay	Biologically active, non-biotinylated, truncated translation products may be generated depending on the codon context or due to a residual RF1 (termination factor 1) activity present in the lysate. If using the biotin residue for detection, choose a biotin position near the N-terminus. If you wish to use a biotin residue incorporated near the C-terminus for detection, purify biotinylated proteins via the incorporated biotin or via an additional C-terminal affinity tag.

		Comments and suggestions
d)	The incorporated biotin residue is not accessible to detection reagents	Try incorporating the biotin moiety at another position. It is possible to add the biotin moiety as the last amino acid of a protein. In this case the amber codon (UAG) is inserted immediately before the termination codon (UAA or UGA). However, labeling efficiencies may be lower near the C-terminus than in the N-terminal region, especially for larger proteins.
Synthesized proteins have an extended C-terminus		
No stop codon present		Ensure that your template contains one of the termination codons UAA or UGA — neither of which is recognized by the BioLys-tRNA — in addition to the UAG codon used for biotin incorporation. This is especially important if a naturally occurring UAG stop codon is used for biotin incorporation. In this case ensure that this UAG is followed by one of the termination codons UAA or UGA.

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Appendix A: Creating a Plasmid DNA Template for Site-Specific Labeling by Site-Directed In Vitro Mutagenesis

Mutations, deletions, or insertions of bases within a DNA plasmid can easily be introduced by PCR amplification of the whole plasmid. This procedure makes use of two user-designed complementary primers of 20 to 40 bp in length, which contain the desired mutation and hybridize to the respective plasmid DNA strands. These primers are used for complete PCR amplification of both strands. After PCR, the complete amplification assay is digested with *Dpn*I to destroy the original, unmutated plasmid DNA. *Dpn*I exclusively digests methylated DNA from *E. coli* whereas unmethylated in vitro synthesized DNA (i.e., DNA amplified by PCR) is not cleaved. Without further purification or ligation, an aliquot of the *Dpn*I digestion is used for direct transformation of competent *E. coli* cells. Colonies growing on selective medium should contain plasmid DNA with the desired mutation.

This in vitro mutagenesis can be performed according to the protocol described below or with any commercially available site-directed mutagenesis kit (e.g., QuikChange® Site-Directed Mutagenesis Kit, Stratagene, cat. no. 200518). Standard protocols (e.g., for transformation and growth of *E. coli* cells) can be found in reference (2).

In vitro mutagenesis primer design

- Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
- Primers may contain point mutations, deletions, or insertions comprising a single or several base pairs.
- Primers should be between 25 and 45 bases in length, and the melting temperature (T_m) of the primers should be greater than or equal to 78°C. The following formula is commonly used for estimating the T_m of primers:

  $T_m = 81.5 + 0.41$ (% GC) – 675/N – % mismatch

  where N is the primer length in bases and values for % GC and % mismatch are whole numbers.
- For calculating Tm for primers intended to introduce insertions or deletions, use this modified version of the above formula:

  $T_m = 81.5 + 0.41$ (% GC) – 675/N

  where N does not include the bases which are being inserted or deleted.
- The desired mutation should be in the middle of the primers with approximately 10–15 bases of correct sequence on both sides.
- Optimally, the primers should have a minimum GC content of 40% and should $T_m$terminate in one or more C or G bases.

- Primers need not be 5' phosphorylated but must be purified either by HPLC or by PAGE. Failure to purify the primers results in a significant decrease in mutagenesis efficiency.

Reagents be supplied by the user

- Plasmid DNA template and gene-specific in vitro mutagenesis primers
- DNA polymerase (e.g., ProofStart DNA Polymerase, cat. no. 202203), reaction buffer, dNTPs
- *Dpn*I restriction enzyme and reaction buffer (e.g., New England Biolabs, cat. no. R0176S)
- Competent *E. coli* cells

Procedure

1. **Thaw 10x ProofStart PCR Buffer, dNTP mix, and primer solutions. Mix the solutions thoroughly before use.**
2. **Prepare a master mix according to Table 7.**

Table 7. PCR components (master mix and template DNA)

Component	Volume/reaction	Final concentration
Master mix		
10x ProofStart PCR Buffer*	5 µl	1x
dNTP (10 mM of each)	1.5 µl	300 µM of each dNTP
Mutagenesis primer		1 µM each primer
ProofStart DNA Polymerase	2 µl	5 units
RNase-free water	variable	—
Template DNA		
Plasmid DNA	variable	~50 ng
Total volume	**50 µl**	**—**

* Contains 15 mM $MgSO_4$

Table 8. PCR cycling conditions for in vitro mutagenesis

	Time	Temp.	Comments
Initial activation step	5 min	95°C	ProofStart DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation	30 s	94°C	
Annealing	1 min	53°C	
Extension	12 min	68°C	
Number of cycles	12–18*		Dependent on the type of mutation (see footnote)

* Use 12 cycles if carrying out a point mutation, 16 cycles for single amino acid changes, or 18 cycles for multiple amino acid deletions or insertions.

3. **Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes.**

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

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

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

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

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

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

   Ensure that the number of cycles used corresponds to the type of mutation being performed (see footnote in Table 8).

8. **After the PCR has cooled, add 10 units *Dpn*I and *Dpn*I reaction buffer to the reaction and incubate for 60 min at 37°C.**

   Add *Dpn*I reaction buffer according to supplier's instructions.

9. **After incubation use an aliquot of the restriction digest to transform competent *E. coli* cells.**

10. **Plate out transformed cells on selective medium and incubate at 37°C.**

11. **Pick several resistant colonies and inoculate LB cultures for DNA minipreps.**

12. **Prepare DNA from minipreps and verify mutation by sequencing.**

Appendix B: Immobilization of Biotinylated Proteins on *Strep*-Tactin or Streptavidin Magnetic Beads

This protocol can be used for the direct immobilization of biotinylated recombinant proteins from in vitro translation reactions.

Equipment and reagents to be supplied by user

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

- In vitro translation reactions containing biotinylated protein
- *Strep*-Tactin Magnetic Beads (QIAGEN, cat. no. 36311) or Streptavidin Magnetic Beads (e.g., Dynabeads M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway). **Note**: The amount of beads added should have a minimum binding capacity of 300 pmol biotin per 25 µl protein synthesis reaction to enable immobilization of the total amount of biotin present in the reaction.
- Magnetic separator (e.g., 12 tube-magnet, QIAGEN cat. no. 36912)
- Suitable binding buffer (depending on the individual requirements of the target protein to be immobilized, e.g. PBS [phosphate buffered saline] or Buffer NP-T [*Strep*-Tactin Beads Wash Buffer])

Buffer compositions are provided in Appendix E on page 50.

Procedure

Equilibration of Streptavidin/*Strep*-Tactin Magnetic Beads

1. **Resuspend magnetic beads by vortexing for 2 s and then immediately pipet an appropriate volume of bead suspension into a 1.5 ml reaction tube.**

   Typically 100 µl of a streptavidin bead suspension or 25 µl of a 10% *Strep*-Tactin Magnetic Bead suspension are sufficient to bind the biotinylated protein.

2. **Place the reaction tube on a magnetic separator for 1 min. Remove supernatant carefully with a pipet.**
3. **Remove the tube from the magnetic separator and pipet 500 µl binding buffer (e.g., PBS or 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. **Repeat step 3.**

Binding of Biotin-tagged proteins

5. **Add 25 µl completed protein synthesis reaction to the beads.**
6. **Add 250 µl binding buffer (e.g. PBS or Buffer NP-T) and bind proteins under gentle shaking (200 rpm on a rotary shaker) for 1 h at 4°C.**

   Binding can be performed overnight if desired. After mixing, tubes may be centrifuged briefly to collect droplets of suspension from the tube caps.

Removal of nonspecifically bound proteins

7. **Place the tube on a magnetic separator for 1 min and remove supernatant with a pipet.**
8. **Remove tube from the magnetic separator, pipet 500 µl PBS (or Buffer NP-T) into the tube, gently vortex the suspension. Place the tube containing the bead suspension on a magnetic separator for 1 min, and remove buffer.**

   Optional: To check the efficiency of immobilization, remove a 200 µl aliquot to a separate tube (see steps 11–15 below).
9. **If necessary, repeat step 8.**
10. **Proceed with desired downstream application.**

To check the efficiency of immobilization, bound proteins may be analyzed by the following procedure

11. **Place the tube containing 200 µl bead suspension from step 8 onto a magnetic separator for 1 min and remove supernatant.**

    Save supernatant for SDS-PAGE analysis.
12. **Add 30 µl SDS-PAGE sample loading buffer (e.g., Roti®-Load 1 [Roth, Karlsruhe, Germany]) to the tube containing the beads.**
13. **Heat the tube for 15 min at 95° to liberate bound proteins and collect beads and liquid by brief centrifugation. Place the tube on a magnetic separator for 1 min and remove supernatant to a clean tube.**
14. **Centrifuge the tube containing the supernatant at full speed in a microcentrifuge for 1 min.**
15. **Load the sample buffer containing the liberated proteins into the wells of an SDS-PAGE minigel.**

16. **Analyze proteins by SDS-PAGE and detect proteins by staining with Coomassie blue.**

    If band smearing occurs during electrophoresis, remove salts by precipitating the supernatant from step 11 with three volumes of ice-cold acetone, incubating for 30 min on ice, and precipitating proteins by centrifugation for 10 min at full speed in a microcentrifuge. Remove the supernatant, allow pellet to dry briefly, and add 25 µl SDS-PAGE sample loading buffer. Heat sample for 15 min at 95°C and analyze by SDS-PAGE.

Appendix C: 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 from 50 µl EasyXpress in vitro translation reactions.

Equipment and reagents to be supplied by user

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

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

Buffer compositions are provided in the Appendix E on page 50.

Procedure

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

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly. 100 µl magnetic bead suspension has a binding capacity of 30 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly. However, use of volumes less than 10 µl 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 µl Buffer NPI-10-T into the tube containing the Ni-NTA Magnetic Agarose Beads and mix by pipetting up and down.**
5. **Pipet the 50 µl in vitro translation reaction into the tube containing the Ni-NTA Magnetic Agarose Beads suspension.**
6. **Mix the suspension gently on an end-over-end shaker for 60 min at 4°C.**
7. **Place the tube on a magnetic separator for 1 min and remove supernatant with a pipet.**

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

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

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

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

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

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

Equipment and reagents to be supplied by user

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

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

Buffer compositions are provided in the Appendix E on page 50.

Procedure

1. **Resuspend Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately pipet 60 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. 60 µl magnetic-bead suspension has a binding capacity of 18 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 100 µl Buffer NPI-10-T into each well. Mix on microplate shaker for 2 s, place the 96-well microplate on a magnet for 1 min, and remove supernatant.**
4. **Pipet a 25 µl in vitro translation reaction aliquot into each well containing Ni-NTA Magnetic Beads.**
5. **Mix the suspension gently on microplate shaker for 60 min at 4°C.**
6. **Place the 96-well microplate on the magnet for 1 min. Carefully remove supernatant with a pipet.**
7. **Pipet 160 µl of Buffer NPI-20-T into each well, mix on a microplate shaker for 2 s, place on the magnet for 1 min, and remove buffer.**
8. **Repeat step 7.**
9. **Add 25 µl of Buffer NPI-250-T to each well, mix on a microplate shaker for 2 s, incubate for 1 min, place on the magnet for 1 min, and collect the eluate.**
10. **Repeat step 9.**

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

Appendix D: 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 to 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 25 to 27.5 µl) without any significant impact on the basal rate of protein synthesis.

Table 9. 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_2MoO_4)	375 µM
Co^{2+} ($CoSO_4$)	375 µM	Se^{4+} (Na_2SeO_3)	1 µM
Cu^{2+} ($CuCl_2$)	90 µM	W^{6+} (Na_2WO_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) using the EasyXpress Protein Synthesis Kit, therefore these values can only be regarded as a guideline for cell-free protein synthesis using the EasyXpress Site-Specific Biotin Kit. It is possible that higher concentrations can be added without compromising protein synthesis.

Table 10. 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
Template concentration	0.1 – 1 µg DNA per 25 µl reaction	0.5 µg per 25 µl reaction
Presence of IPTG (for IPTG-inducible plasmids)		1 mM final concentration
Reaction dilution	1.1x to 1.6x (add 2.5 – 15 µl water to reaction)	1.4x (add 10 µl water to reaction)
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 9, page 48	
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)	Up to 5.4 µl per 25 µl reaction (pH 6.5–7.4)	
Basic buffer (0.25 M KOH)	Up to 2.4 µl per 25 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 E: Buffers and Reagents

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

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

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

Adjust pH to 8.0 using NaOH.

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

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

Adjust pH to 8.0 using NaOH.

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

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

Adjust pH to 8.0 using NaOH.

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

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

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

Adjust pH to 8.0 using NaOH.

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

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

Adjust pH to 8.0 using NaOH.

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

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

Adjust pH to 8.0 using NaOH.

Buffers for immobilizing biotinylated proteins using *Strep*-Tactin Magnetic Beads

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

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

Adjust pH to 8.0 using NaOH.

Ordering Information

Product	Contents	Cat. no.
EasyXpress Site-Specific Biotin Kit	For 5 x 25 µl reactions: *E. coli* extract, reaction buffer, RNase-Free Water, biotinyl-lysyl tRNA (amber), and positive-control DNA	32602
EasyXpress 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 in vitro translation reactions or *E. coli*: 25 µg vector DNA	32733
Related products		
QIAgenes Expression Kit *E. coli*	QIAgenes Expression Construct (10 µg), QIAgenes *E. coli* Positive Control (10 µg), Penta·His Antibody, BSA-free (3 µg), 4 Ni-NTA Spin Columns	Varies
EasyXpress Protein Synthesis Kit (5)	For 5 x 50 µl reactions: *E. coli* extract, reaction buffer, RNase-Free Water, and positive-control DNA	32501
EasyXpress Protein Synthesis Kit (20)	For 20 x 50 µl reactions: *E. coli* extract, reaction buffer, RNase-Free Water, and positive-control DNA	32502
EasyXpress Protein Synthesis Maxi Kit	For reactions up to 4000 µl: 4 x 350 µl *E. coli* extract, reaction buffer, RNase-Free Water, and positive-control DNA	32506
EasyXpress Random Biotin Kit	For 60 x 50 µl reactions: biotinyl-lysyl tRNA (Phe)	32612
EasyXpress Protein Synthesis Mega Kit	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o methionine, methionine, RNase-Free Water, gel-filtration columns, and reaction flasks	32516

Ordering Information

Product	Contents	Cat. no.
EasyXpress NMR Protein Synthesis Kit	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o Arg, Lys, Ser, Thr, Val (supplied as individual amino acids), RNase-Free Water, gel-filtration columns, and reaction flasks	32526
EasyXpress NMR Protein Synthesis Kit –X*	For 2 x 5 ml reactions: *E. coli* extract, amino acid mix w/o X, reaction buffers, RNase-Free Water, gel-filtration columns, and reaction flasks	Varies
EasyXpress Insect Kit II (5)	For 5 x 50 µl reactions: *Spodoptera frugiperda* 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: *Spodoptera frugiperda* 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 Protein Synthesis Insect Kit	32713
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

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

Ordering Information

Product	Contents	Cat. no.
Ni-NTA Agarose (25 ml)	25 ml nickel-charged resin (max. pressure: 2.8 psi)	30210
Strep-Tactin Superflow (2 ml)	For batch and HPLC purification of *Strep*-tagged proteins: 2 ml *Strep*-Tactin-charged Superflow (max. pressure: 140 psi)	30001
Polypropylene Columns (5 ml)	50/pack, 5 ml capacity	34964
QIArack	1 rack for holding gel-filtration columns or affinity-resin filled polypropylene columns	19015
Ni-NTA Magnetic Agarose Beads (2 x 1 ml)	2 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36111
Strep-Tactin Magnetic Beads (2 x 1 ml)	For micro-scale purification of *Strep*-tagged proteins: 2 x 1 ml *Strep*-Tactin-charged magnetic agarose beads (10% suspension)	36311
Factor Xa Protease*	400 units Factor Xa Protease (2 units/µl)	33223
Xa Removal Resin	2 x 2.5 ml Xa Removal Resin, 3 x 1.9 ml 1 M Tris-Cl, pH 8.0	33213
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
Strep-tag Antibody (100 ug)	Mouse monoclonal antibody that recognizes the *Strep*-tag II epitope; lyophilized, for 1000 ml working solution	34850
Streptavidin-R-PE	250 µg Streptavidin-R-phycoerythrin Conjugate, 1 mg/ml	922721
6xHis Protein Ladder	6xHis-tagged marker proteins (lyophilized, for 50-100 lanes on western blots)	34705

* Not available in all countries, please inquire.

Ordering Information

Product	Contents	Cat. no.
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
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

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Trademarks: QIAGEN®,QIAprep®, EasyXpress®, HiSpeed®, LiquiChip®, MinElute®, Omniscript®, SensiScript® (QIAGEN Group); BIACORE® (Biacore AB); Coomassie® (ICI Organics Inc.) QuikChange® (Stratagene, an Agilent Technologies company); Ready Protein+™(Beckman Coulter, Inc.), *Strep*-tag™, *Strep*-Tactin™ (IBA GmbH); TAGZyme (UNIZYME), Tween® (ICI Americas Inc); xMAP™ (Luminex Corp.)

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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.

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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).

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