

# EasyXpress® Insect Cell Protein Synthesis Handbook

For in vitro synthesis of proteins with  
posttranslational modifications using  
insect-cell lysates

EasyXpress Insect Kit II

EasyXpress pIX4.0 Vector

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

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## Kit Contents

<b>EasyXpress Insect Kit II</b>	<b>20 x 50 µl reactions</b>	<b>5 x 50 µl reactions</b>
<b>Cat. no.</b>	<b>32562</b>	<b>32561</b>
EasyXpress Insect Kit II Extract (colorless snap-cap)	4 x 100 µl	1 x 100 µl
EasyXpress Insect Kit II Reaction Buffer (blue screw-cap)	1 x 100 µl	1 x 25 µl
EasyXpress Insect Kit II Energy Mix (red screw-cap)	1 x 100 µl	1 x 25 µl
RNase-Free Water (colorless screw-cap)	1 x 1.9 ml	1 x 1.9 ml
EasyXpress Insect Kit II Positive Control DNA (violet screw-cap)	1 x 25 µl	1 x 6.25 µl
EasyXpress Insect Kit II 5x Transcription Buffer (yellow screw-cap)	1 x 100 µl	1 x 25 µl
EasyXpress Insect Kit II 5x NTP Mix (green screw-cap)	1 x 100 µl	1 x 25 µl
EasyXpress Insect Kit II 20x Enzyme Mix (orange screw-cap)	1 x 25 µl	1 x 6.25 µl
DyeEx® 2.0 Spin Columns	20	5
Handbook	1	1

<b>EasyXpress pIX4.0 Vector</b>	<b>Cat. no. 32713</b>
EasyXpress pIX4.0 Vector (white screw-cap)	25 µg (0.5 µg/µl)
Handbook	1

<b>EasyXpress Random Biotin Kit</b>	<b>For 60 x 50 µl reactions</b>
<b>Cat. no.</b>	<b>32612</b>
EasyXpress Biotinyl-Lysyl tRNA (Phe)	4 x 15 µl
Product Sheet	1

## Storage

The EasyXpress Insect Kit II is shipped on dry ice.

**5x Transcription Buffer**, **5x NTP Mix**, and **20x Enzyme Mix** must be stored at  $-20^{\circ}\text{C}$  upon arrival.

**EasyXpress Insect Extract**, **EasyXpress Insect Reaction Buffer**, and **EasyXpress Insect Positive Control DNA** must be stored at  $-70^{\circ}\text{C}$  upon arrival.

Once thawed, EasyXpress Insect Extract should be stored on ice and used within 4 hours.

The **EasyXpress pIX4.0 Vector** is shipped on dry ice and must be stored at  $-20^{\circ}\text{C}$  upon arrival.

**DyeEx 2.0 Spin Columns** should be stored dry at room temperature ( $15\text{--}25^{\circ}\text{C}$ ). For longer storage, these kits can be stored at  $2\text{--}8^{\circ}\text{C}$ . Do not freeze.

When stored under the above conditions and handled correctly, the EasyXpress Insect Kit II can be stored for at least 6 months without showing any reduction in performance. The EasyXpress pIX4.0 Vector can be stored for at least 1 year.

**The EasyXpress Random Biotin Kit** is shipped on dry ice and should be stored at  $-70^{\circ}\text{C}$ . Once thawed, Biotinyl-Lysyl tRNA should be stored on ice and quickly returned to a  $-70^{\circ}\text{C}$  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 6 months without showing any reduction in performance.

## Product Use Limitations

EasyXpress Kits are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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](http://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](http://www.qiagen.com/Support) or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit [www.qiagen.com](http://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/support/MSDS.aspx](http://www.qiagen.com/support/MSDS.aspx) 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 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. Proteins produced by cell-free expression can be used for a wide variety of downstream applications; including activity assays, interaction studies (protein–protein, protein–ligand, and protein–DNA), small-molecule inhibition, and the expression and analysis of open reading frames and expression constructs.

A broad range of eukaryotic proteins require posttranslational modifications such as phosphorylation, glycosylation, or signal peptide cleavage to display full functional activity. Eukaryotic cell-free expression systems provide the possibility to synthesize eukaryotic proteins with posttranslational modifications and are especially useful for expression and analysis of human proteins with native structure and function.

The EasyXpress Insect Kit II, a new eukaryotic cell-free expression system, enables expression of eukaryotic proteins — including membrane proteins — with posttranslational modifications. In contrast to many rabbit-reticulocyte lysate (RRL)-based systems, the insect-cell extract does not require any additives to display full functionality. In addition, the EasyXpress pIX4.0 Vector has been developed for generation of optimal expression templates to be used with the EasyXpress Insect Kit II.

## Principle and procedure

The EasyXpress Insect Kit II uses highly productive insect cell lysates obtained from a *Spodoptera frugiperda* cell line, which contain all translational machinery components (i.e., ribosomes, ribosomal factors, tRNAs, aminoacyl-tRNA synthetases, etc.) required for efficient protein synthesis. In addition, the lysates contain functional organellar membrane fractions, whose activity is required for posttranslational modification of eukaryotic proteins, including membrane proteins (Figures 1 and 2). It is a linked transcription–translation system (see flowchart, page 11).

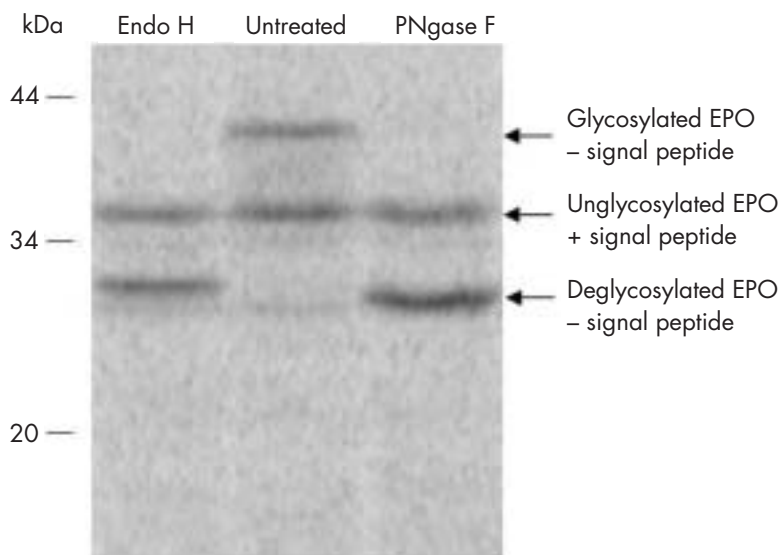
In the in vitro transcription reaction, high-quality capped mRNA is produced using linearized or circular plasmid DNA or PCR products containing a T7 promoter. In the linked cell-free expression reaction, the capped mRNA is used as template to express active full-length proteins.

Using the EasyXpress Insect Kit, up to 40 µg/ml functionally active posttranslationally modified protein can be synthesized within 3.5 hours (1).

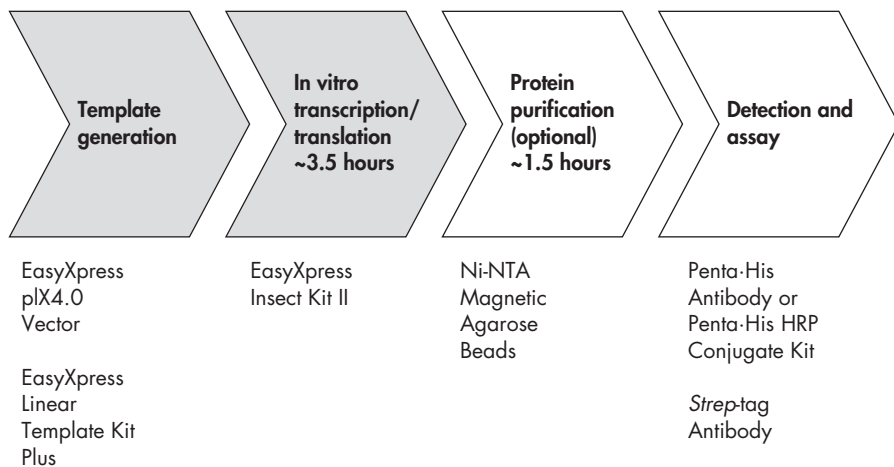




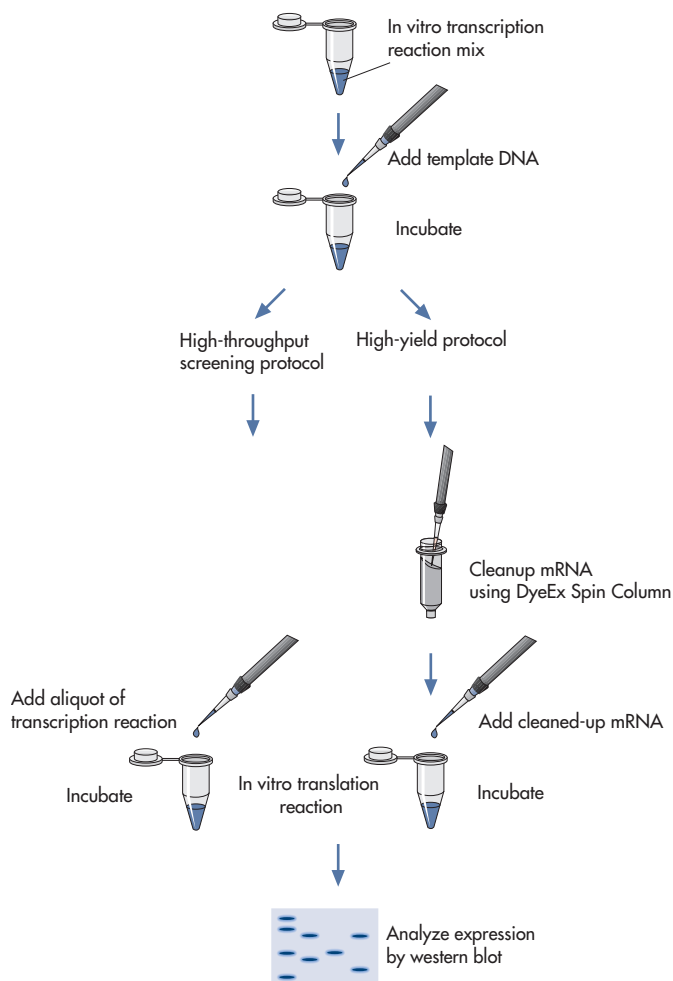
**Figure 1. Proteins successfully expressed using the EasyXpress Insect Kit II.** 6xHis-tagged protein kinases and cannabinoid receptor (CNR1) were visualized using the Penta-His HRP Conjugate. Clotting factors and OGCP (mitochondrial 2-oxoglutarate/malate carrier) were synthesized in duplicate reactions using  $^{14}\text{C}$ -labeled amino acids and visualized using a PhosphorImager<sup>®</sup>.



**Figure 2. Efficient glycosylation of erythropoietin.** The glycoprotein Erythropoietin (EPO) was synthesized using the EasyXpress Insect Kit II in the presence of  $^{14}\text{C}$ -labeled amino acids. To remove the glycan moieties from the synthesized glycoproteins, aliquots of the synthesis reactions were incubated either in the presence of endoglycosidase H (**Endo H**) or peptide N-glycosidase F (**PNGase F**). After separation by SDS-PAGE, proteins were visualized using a PhosphorImager. Removal of the glycan moieties increases the electrophoretic mobility of the protein compared to the glycosylated form.



## EasyXpress Protein Synthesis Procedure



# DNA Templates

The EasyXpress Insect Kit II can be used to express proteins from a variety of DNA templates. Templates must contain a T7 promoter upstream of the coding sequence. Suitable DNA templates include circular and linearized plasmid DNA, and PCR products (e.g., generated using the EasyXpress Linear Template Kit Plus, see page 13).

## Minimum template requirements

DNA templates must contain the T7 promoter (Figure 3) for transcription. A stretch of at least 5 base pairs should be placed upstream of the promoter. The sequence of the transcribed mRNA must begin with at least one G. The 5' untranslated region (5'-UTR) must not contain an ATG triplet in any reading frame. Strong secondary structures within the 5'-UTR should be avoided. The translation start codon must be ATG and the translation stop codon must be TAA, TAG, or TGA. When using circular plasmid DNA as template, the plasmid must contain a T7 terminator.

For optimal efficiency of transcription and translation we strongly recommend using the cloning and expression vector EasyXpress pIX4.0 (cat. no. 32713), see Figure 5, page 15.

5' . . . **XXXXX****TAATACGACTCACTATAG**           . . . 3'

Figure 3. Sequence of T7 promoter (bold) and transcription start (underlined).

## Plasmid DNA

Greatest yields of capped mRNA, and consequently, high protein yields 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 (2) may be sufficiently pure, but DNA must be free of RNases.

To achieve optimal protein yields we recommend linearization of expression plasmid DNA prior to in vitro transcription. The plasmid DNA must be linearized using a restriction enzyme that cuts downstream of the insert to be transcribed. We recommend using restriction enzymes that produce blunt ends. Restriction enzymes that produce 3' overhangs should be avoided.

Following digestion, linearized DNA should be cleaned up, for example using the QIAquick® Nucleotide Removal Kit (QIAGEN, cat. no. 28304) and resuspended in RNase-free water.

For linearized DNA encoding mRNAs of less than 1500 bases, 1 µg linearized DNA template should be added to each 25 µl in vitro transcription reaction at a concentration of 0.2 µg/µl. In some cases, the yield of protein synthesis can be improved by increasing the DNA amount to 1.5 µg or decreasing the amount to 0.5 µg per 25 µl transcription reaction. Alternatively, the volume of cleaned up mRNA (see Table 4 on page 24) added to the translation reaction can be lowered to 6 µl or increased to 20 µl.

For linearized DNA encoding mRNAs of greater than 1500 bases, 1.5 µg linearized DNA template should be added to each 25 µl in vitro transcription reaction at a concentration of 0.3 µg/µl. In some cases, the yield of protein synthesis can be improved by increasing the DNA amount to 2.0 µg or decreasing the amount to 1.0 µg per 25 µl transcription reaction. Alternatively, the volume of cleaned up mRNA (see table 4 on page 24) added to the translation reaction can be lowered to 6 µl or increased to 20 µl.

If using circular plasmid DNA, 1.5 µg should be added to each 25 µl in vitro transcription reaction.

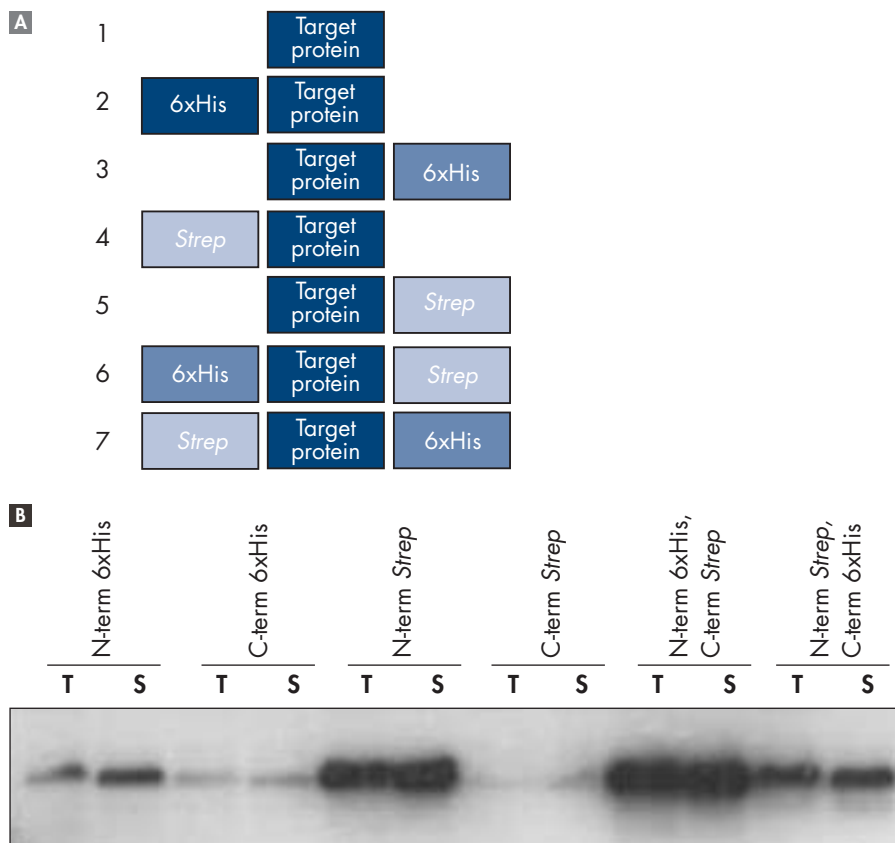
### PCR products

If PCR products are added to the in vitro transcription reaction, we recommend that they are generated using the EasyXpress Linear Template Kit Plus (cat. no. 32723). PCR products can be added directly to the in vitro transcription reactions without further cleanup. The amount of PCR product added to each 25 µl in vitro transcription reaction should be 500 ng. **Note:** XE-solution provided with the EasyXpress Linear Template Kit Plus **should not be added** to the in vitro transcription reaction.

The *EasyXpress Linear Template Kit Plus Handbook* gives comprehensive and detailed information on producing PCR products suitable for use as expression constructs with EasyXpress Protein Synthesis Kits.

### 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 4A). 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.



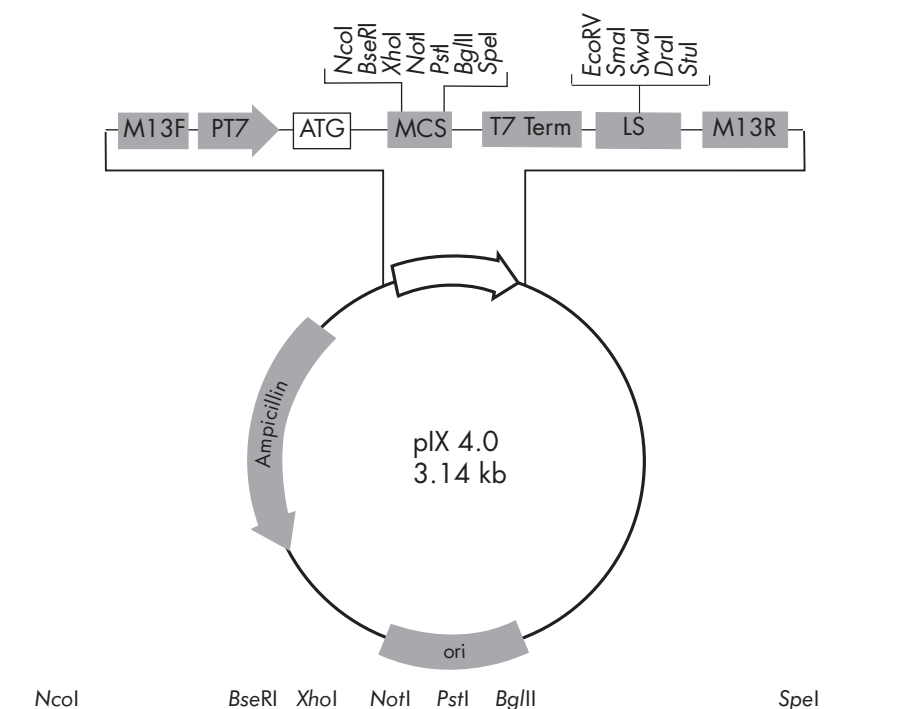
**Figure 4. Expression analysis reveals the optimal construct.** PCR products generated using the EasyXpress Linear Template Kit Plus and carrying the indicated tag(s) **A** are added to small-scale IVT reactions. **B** TFII $\alpha$  variants were synthesized using the EasyXpress Insect Kit II and separated by SDS-PAGE, transferred to a membrane, and visualized using a mixture of Anti-His Antibodies and *Strep*-tag antibodies and chemiluminescent detection. **T**: total protein; **S**: soluble fraction.

The screening of such constructs in small-scale cell-free expression (IVT) reactions using the insect-cell based EasyXpress Insect Kit II (or *E. coli*-based EasyXpress Protein Synthesis Kits) 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 48). 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.

# EasyXpress pIX4.0 Vector

The EasyXpress pIX4.0 Vector is designed for high-level expression of proteins using the EasyXpress Insect Kit II. This high-copy plasmid has the following features:

- Efficient initiation of translation through a T7 promoter element
- Optimized 3' UTR, including a T7 terminator, and an optimally positioned linearization site
- Multiple cloning site and translational stop codons in all reading frames for convenient preparation of expression constructs
- Optimized 3' UTR combined with T7 terminator for generation of stabilized RNA protected from degradation by exonucleolytic nucleases
- Site for plasmid linearization consisting of multiple restriction sites for blunt end linearization for effective in vitro transcription
- $\beta$ -lactamase gene conferring resistance to ampicillin



CC**ATG**GGAGACCCCTCCTCGAGCGGCCGCCTGCAGATCTAAATAATAAGTAATTAAGTAGT  
 GGTACCCCTCTGGGGAGGAGCTCGCCGGCGGACGCTAGATTTATTATTTCATTAATTGATCA  
 Met Gly Asp Pro Ser Ser Ser Gly Arg Leu Gln Ile

**Figure 4. The pIX4.0 vector.** M13F: M13 forward, PT7: T7 promoter, MCS: multiple cloning site, T7 term: T7 terminator, LS: Linearization site, M13R: M13 reverse, ori: origin of replication.

## Designing primers for cloning into the EasyXpress pIX4.0 Vector

Comprehensive instructions for cloning protein-coding sequences into the EasyXpress pIX4.0 Vector can be found in Appendices E and F on pages 41–51.

## 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 [<sup>35</sup>S] methionine or [<sup>14</sup>C] 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

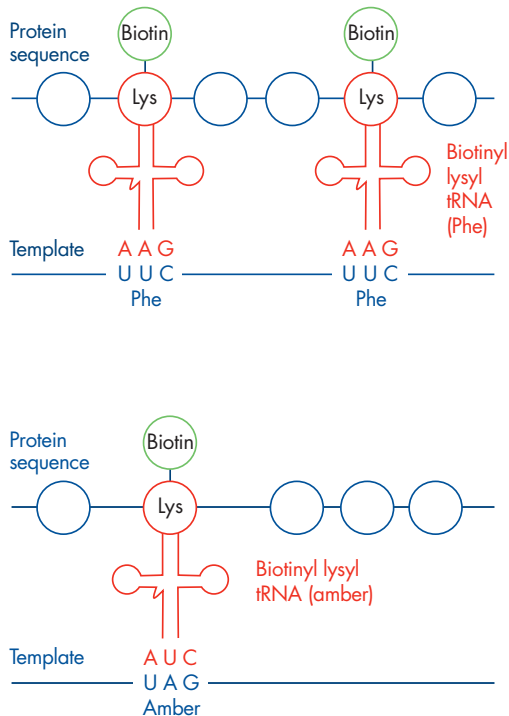
The EasyXpress Random Biotin Kit comprises a synthetic tRNA aminoacylated with lysine labeled at the epsilon position with biotin and carrying a phenylalanine GAA anticodon. This tRNA directs the incorporation of a biotin residue at phenylalanine UUC codons (Figure 6). To incorporate biotin into a recombinant 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 7). 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).

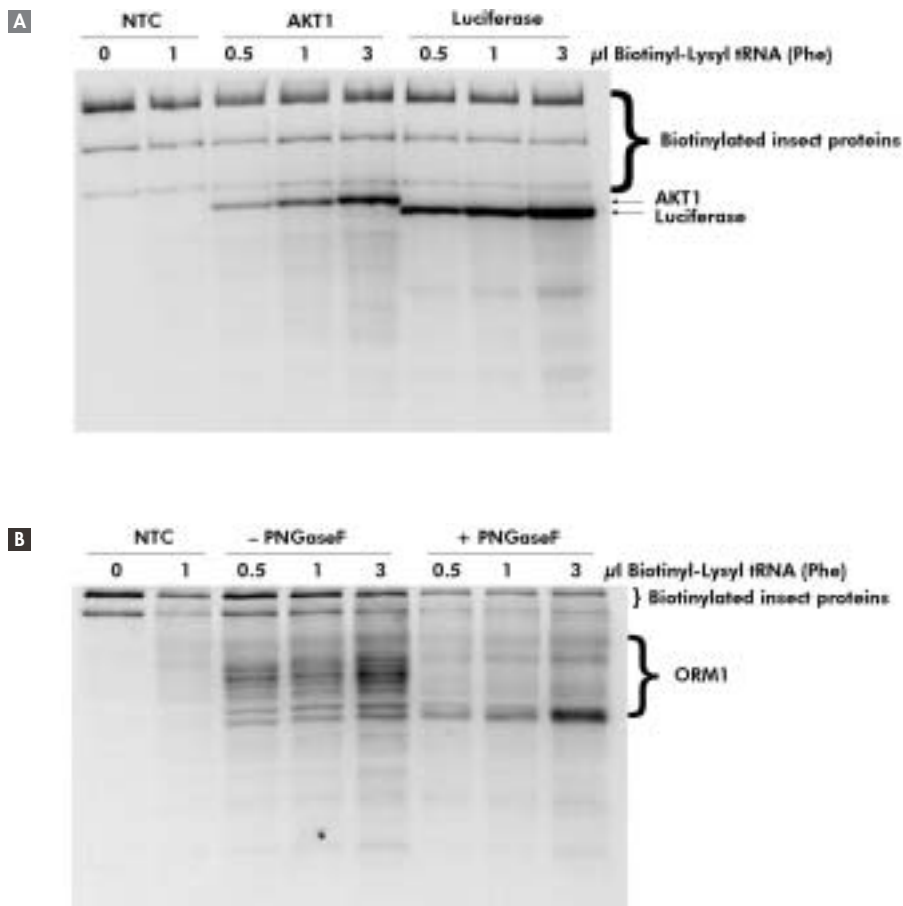


**Table 1. Biotinyl-lysyl tRNA in the EasyXpress Random Biotin Kit**

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



**Figure 6. Schematic representation of biotin incorporation using the EasyXpress Random Biotin Kit.**



**Figure 7. Synthesis of biotinylated recombinant proteins with the EasyXpress Insect Kit II.** **A** Human RAC-alpha serine/threonine kinase (**AKT1**) and **luciferase** (control protein supplied with the kit) were synthesized using the EasyXpress Insect Kit II and the indicated amount of biotinyl-lysyl tRNA (Phe). **NTC**: no template control. Proteins were separated by electrophoresis in a 10% SDS-PAGE Mini Gel. Detection of biotinylated proteins was performed using streptavidin-peroxidase following western blotting. **B** Human alpha-1-acid glycoprotein (**ORM1**) was synthesized using the EasyXpress Insect Kit II and the indicated amount of biotinyl-lysyl tRNA (Phe). **NTC**: no template control. To demonstrate the extent of glycosylation, reaction aliquots were removed and treated with the deglycosidase PNGaseF. Aliquots of treated and untreated samples were separated by electrophoresis in a 15% SDS-PAGE Mini Gel. Detection of biotinylated proteins was performed using streptavidin-peroxidase following western blotting.

# Protocol: Protein Synthesis Using the EasyXpress Insect Kit II

This protocol is suitable for the *in vitro* synthesis of recombinant proteins with posttranslational modifications using the EasyXpress Insect Kit II (cat. nos. 32561 and 32562). To facilitate sample analysis, proteins can be labeled with biotin using the EasyXpress Random Biotin Kit (cat. no. 32612). Templates suitable for use as expression constructs can be generated using the EasyXpress Linear Template Kit Plus (cat. no. 32723).

The protocol is divided into two sections: *in vitro* transcription and *in vitro* translation. Using the positive control DNA template, the high-yield protocol delivers up to 40 µg active luciferase per ml reaction. A faster high-throughput version of the translation protocol is provided in Appendix A, page 29. In this protocol, the transcription reaction is added directly to the translation reaction without cleanup: however, protein yields are lower.

## Materials and reagents to be supplied by the user

- DNA (linearized or circular plasmid, or PCR product) encoding the protein of interest. The plasmid must contain a T7 promoter (see page 12)
- Thermomixer® (Eppendorf, Hamburg, Germany)

## 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.
- EasyXpress Insect Extract is provided as individual 100 µl aliquots in single tubes. Once thawed, store EasyXpress Insect Extract on ice and use within 4 hours. Refreeze the extracts in liquid nitrogen. Do not refreeze and thaw more than four times.
- The recommended incubation temperature for transcription is 37°C, the incubation temperature for protein synthesis is 27°C.
- We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at -20°C.
- For multiple reactions, prepare a master mix without template, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the template.
- 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.

## Additional points to consider when producing biotinylated proteins

- Do not add Biotinyl-Lysyl-tRNA to a master mix. This may result in lower amounts of biotin incorporation due to deacylation of the tRNA carrying the biotinylated residue. Start the reaction by adding Biotinyl-Lysyl-tRNA to the reaction 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 template and the Biotinyl-Lysyl-tRNA.
- Once thawed, Biotinyl-Lysyl tRNA should be stored on ice and quickly returned to a  $-70^{\circ}\text{C}$  freezer after use. Do not thaw and refreeze more than four times.

## Procedure

### In vitro transcription reaction

1. **Thaw and store 5x Transcription Buffer, 5x NTP Mix, 20x Enzyme Mix, RNase-Free Water, and EasyXpress Insect Positive Control DNA on ice.**

Before use, gently vortex and briefly centrifuge each tube to ensure homogeneity of solutions.

2. **Pipet together the components of the three transcription reactions shown in Table 2 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes at room temperature (15–25°C). Label each tube clearly.**

It is important to add the reaction components in the order shown in Table 2.

3. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
4. **Incubate the in vitro transcription reactions for 120 min at 37°C.**
5. **Centrifuge the reactions for 1 min at 12,000 x g at room temperature (15–25°C). Pipet the reaction supernatants into clean DNase- and RNase-free 1.5 ml microcentrifuge tubes and keep at room temperature (15–25°C). Label each tube clearly.**

**Table 2. Pipetting scheme for transcription reactions**

Reagent	Target protein template reaction	Positive control reaction	No template control reaction
RNase-Free water	8.75 µl	8.75 µl	13.75 µl
5x Transcription Buffer	5 µl	5 µl	5 µl
5x NTP Mix	5 µl	5 µl	5 µl
20x Enzyme Mix	1.25 µl	1.25 µl	1.25 µl
EasyXpress Insect Positive Control DNA	—	5 µl	—
DNA	5 µl*	—	—
<b>Total</b>	<b>25 µl</b>	<b>25 µl</b>	<b>25 µl</b>

\* For **linearized DNA encoding mRNAs of less than 1500 bases**, 1 µg linearized DNA template should be added to each 25 µl in vitro transcription reaction at a concentration of 0.2 µg/µl.

For **linearized DNA encoding mRNAs of greater than 1500 bases**, 1.5 µg linearized DNA template should be added to each 25 µl in vitro transcription reaction at a concentration of 0.3 µg/µl.

For **circular plasmid DNA**, 1.5 µg template should be added to each 25 µl reaction.

For **PCR products**, 500 ng template should be added to each 25 µl reaction.

**6. For the high-throughput screening protocol, proceed using the protocol in Appendix A on page 29. For the high-yield protocol proceed with step 7 below.**

We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at –20°C.

**In vitro translation reaction using the high-yield protocol**

In this part of the protocol, mRNA is cleaned up using a DyeEx gel-filtration spin column before addition to the translation reaction.

**Important note before starting**

All centrifugation steps are performed at 750 x g in a conventional microcentrifuge. The appropriate speed for individual centrifuges can be calculated as follows:  
 $\text{rpm} = 1000 \times \sqrt{750/1.12 \text{ r}}$  (r = radius of rotor in mm).

**Table 3. Examples of suitable microcentrifuges**

<b>Microcentrifuge</b>	<b>Speed corresponding to 750 x g</b>
Eppendorf® Centrifuge 5415C	3000 rpm
Eppendorf Centrifuge 5417C	2700 rpm
Heraeus Biofuge 15	2800 rpm
Hettich Mikro 24-48	2630 rpm
Beckman GS15R	2100 rpm
Hettich Mikro EBA12	2700 rpm

**Procedure**

- 7. Gently vortex the DyeEx spin column to resuspend the resin.**
- 8. Loosen the cap of the column a quarter turn.**  
This is necessary to avoid a vacuum developing inside the spin column.
- 9. Snap off the bottom closure of the spin column (Figure 8), and place the spin column into a DNase- and RNase-free 2 ml microcentrifuge tube.**

**Figure 8. Snapping off the bottom closure of the DyeEx 2.0 spin column (do not screw).**

- 10. Centrifuge for 3 min at the calculated speed (see Table 3).**

11. **Carefully transfer the spin column to a clean centrifuge tube. Slowly apply the 20  $\mu$ l in vitro transcription reaction to the gel bed (Figure 9).**

Pipet the in vitro transcription reaction directly onto the center of the slanted gel-bed surface (Figure 9). Do not allow the reaction mixture or the pipet tip to touch the sides of the column.

The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip. It is not necessary to replace the lid on the column. **12.**

**Centrifuge for 3 min at the calculated speed (see Table 3).**

13. **Remove the spin column from the microcentrifuge tube. Keep the eluate at room temperature and proceed immediately with the in vitro translation reaction.**

The eluate contains the purified RNA.



Figure 9. Instructions for sample application to the DyeEx 2.0 spin column.

14. **Thaw and store EasyXpress Insect Reaction Buffer, EasyXpress Insect Extract, EasyXpress Energy Mix, and RNase-Free Water on ice. If using the EasyXpress Random Biotin Kit for cotranslational labeling, thaw EasyXpress Biotinyl-Lysyl tRNA (Phe) on ice.**
15. **Pipet together the components of the three translation reactions shown in Table 4 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes. Label each tube clearly.**

It is important to add the reaction components in the order shown in Table 4. **Note:** if template mRNA has been stored overnight at  $-20^{\circ}\text{C}$ , thaw at room temperature ( $15\text{--}25^{\circ}\text{C}$ ) and vortex briefly before adding to the translation reaction.

**Table 4. Pipetting scheme for high-yield in vitro translation reactions**

<b>Reagent</b>	<b>Target protein synthesis reaction</b>	<b>Positive control reaction</b>	<b>No template control reaction</b>
RNase-Free Water	Add to 50 $\mu$ l	Add to 50 $\mu$ l	Add to 50 $\mu$ l
EasyXpress Insect Reaction Buffer	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
EasyXpress Insect Extract	20 $\mu$ l	20 $\mu$ l	20 $\mu$ l
Template mRNA *	12 $\mu$ l	12 $\mu$ l	12 $\mu$ l
Insect EasyXpress Energy Mix	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
Biotinyl-Lysyl-tRNA (Phe) <sup>†</sup>	1–3 $\mu$ l	1–3 $\mu$ l	1–3 $\mu$ l
<b>Total</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>

\* From step 13. Use an aliquot of the DyeEx spin column flow-through from the respective transcription reaction.

<sup>†</sup> **Note:** It is important that the EasyXpress Biotinyl-Lysyl tRNA is added as the last reaction component.

- Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
- Incubate the in vitro translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.**
- If producing biotinylated proteins, add 1  $\mu$ l EasyXpress Biotinyl-Lysyl-tRNA to each reaction 10 min after starting the translation reaction.**

It is important that the EasyXpress Biotinyl-Lysyl tRNA is added as the last reaction component. To enhance signal intensity in detection procedures, the proportion of incorporated biotin can be increased by adding up to 3  $\mu$ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) per reaction.

**19. Proceed with sample analysis.**

Biotin labeled proteins can be detected after separation on SDS-PAGE and subsequent blotting by colorimetric or chemiluminescent visualization with Streptavidin-AP or Streptavidin-HRP. Usually 1–5  $\mu$ l of the in vitro translation reaction is sufficient for one gel lane.



# Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: [www.qiagen.com/FAQ/FAQList.aspx](http://www.qiagen.com/FAQ/FAQList.aspx) . The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit [www.qiagen.com](http://www.qiagen.com) ).

## Comments and suggestions

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### No target 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. Generate PCR products using the EasyXpress Linear Template Kit Plus.   |
| 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 (see "Minimum template requirements", page 12). Ensure that the expression construct contains a T7 promoter.  |
| 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.<br><br>If the protein to be expressed already contains a tag, move the tag to the opposite terminus.   |
| e) Coding sequence not optimal for insect cell-based expression systems             | Adapt DNA sequence to optimize expression in insect cell-based systems. QIAGENes offer human gene constructs whose sequences are optimized for expression in eukaryotic systems (see <a href="http://www.qiagen.com/goto/qiagenes">www.qiagen.com/goto/qiagenes</a> ). |

### Low expression yield

- 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. Generate PCR products using the EasyXpress Linear Template Kit Plus.
- Increase or reduce the amount of DNA in the in vitro transcription reaction by 0.5 µg per 25µl reaction. Alternatively titrate the volume of cleaned up mRNA added to the in vitro translation reaction between 6 and 20 µl, if using the high-yield protocol.
- b) GC-rich mRNA
- Denature the template mRNA prior to in vitro translation at 65°C for 3 min and immediately cool in an ice-water bath. This may increase the efficiency of translation, especially of GC-rich mRNA, by destroying local regions of secondary structure.
- c) mRNA has precipitated
- Ensure that mRNA is kept at room temperature (15–25°C) after transcription and after cleanup. If stored on ice, mRNA may precipitate, resulting in lower yields.
- If template mRNA has been stored overnight at –20°C, thaw at room temperature (15–25°C) and vortex briefly before adding to the translation reaction.
- d) Coding sequence not optimal for insect cell-based expression systems
- Adapt DNA sequence to optimize expression in insect cell-based systems. QIAGENes offer human gene constructs whose sequences are optimized for eukaryotic expression (see [www.qiagen.com/goto/qiagenes](http://www.qiagen.com/goto/qiagenes) ).

### Poor incorporation of biotin/weak or no signal on western blot

- |   |  |
|---|--|
| a) Bio-Lys tRNA added to master mix                 | Do not add Bio-Lys 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 Bio-Lys tRNA to the master mix as recommended in the protocol.  |
| b) 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 Bio-Lys 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. |
| c) 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](http://www.qiagen.com/RefDB/search.asp) or contact QIAGEN Technical Services or your local distributor.

### Cited references

- 1) Kubick, S., Merk, H., Stiege, W., von Groll, U., Drees, J., and Schaefer, F. Insect-cell based in vitro synthesis of posttranslationally modified proteins. QIAGEN News 2004. 41.
- 2) Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning — A laboratory Manual*. 2<sup>nd</sup> Ed. Cold Spring Harbor. Cold Spring Harbor Laboratory Press.

## Appendix A: In Vitro Translation Reaction Using the High-Throughput Screening Protocol

1. Without further processing, use 5  $\mu$ l of each in vitro transcription reaction for an in vitro translation reaction.
2. Thaw and store EasyXpress Insect Reaction Buffer, EasyXpress Insect Extract, EasyXpress Energy Mix, and RNase-Free Water on ice. If using the EasyXpress Random Biotin Kit for cotranslational labeling, thaw EasyXpress Biotinyl-Lysyl tRNA (Phe) on ice.
3. Pipet together the components of the three translation reactions shown in Table 5 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes. Label each tube clearly.

It is important to add the reaction components in the order shown in Table 5.

4. Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.
5. Incubate the in vitro translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.
6. Proceed with sample analysis.

Biotin labeled proteins can be detected after separation on SDS-PAGE and subsequent blotting by colorimetric or chemiluminescent visualization with Streptavidin-AP or Streptavidin-HRP. Usually 1–5  $\mu$ l of the in vitro translation reaction is sufficient for one gel lane.

**Table 5. Pipetting scheme for high-throughput screening in vitro translation reactions**

<b>Reagent</b>	<b>Target protein synthesis reaction</b>	<b>Positive control reaction</b>	<b>No template control reaction</b>
RNase-Free Water	Add to 50 $\mu$ l	Add to 50 $\mu$ l	Add to 50 $\mu$ l
EasyXpress Insect Reaction Buffer	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
EasyXpress Insect Extract	20 $\mu$ l	20 $\mu$ l	20 $\mu$ l
Template mRNA *	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
Insect EasyXpress Energy Mix	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
When producing randomly biotinylated proteins:			
Biotinyl-Lysyl-tRNA (Phe) <sup>†</sup>	1–3 $\mu$ l	1–3 $\mu$ l	1–3 $\mu$ l
<b>Total</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>

\* From step 5 of the transcription protocol on page 20. Use an aliquot of the respective transcription reaction.

<sup>†</sup> **Note:** It is important that the EasyXpress Biotinyl-Lysyl tRNA is added as the last reaction component. To enhance the specific signal intensity and to minimize background labeling (thereby optimizing signal-to-background ratio), start translation reactions before addition of the EasyXpress Biotinyl-Lysyl-tRNA and add EasyXpress Biotinyl-Lysyl-tRNA 10 minutes after starting the translation reaction. To enhance signal intensity in detection procedures, the proportion of incorporated biotin can be increased by adding up to 3  $\mu$ l EasyXpress Biotinyl-Lysyl-tRNA (Phe) per reaction. In cases where EasyXpress Biotinyl-Lysyl tRNA is added to a master mix, work quickly to avoid deacylation of the EasyXpress Biotinyl-Lysyl tRNA.

## Appendix B: Scaling Up Protein Synthesis Using the EasyXpress Insect Kit II

This protocol is suitable for scaling up *in vitro* synthesis of recombinant proteins with posttranslational modifications using the EasyXpress Insect Kit II in a 900 µl translation reaction volume. If smaller reactions are desired, scale down the volumes of the reaction components accordingly.

The protocol is divided into two sections, *in vitro* transcription and *in vitro* translation. Using the positive control DNA template, the high-yield protocol delivers up to 40 µg active luciferase per ml reaction.

### Materials and reagents to be supplied by the user

- DNA (linearized or circular plasmid, or PCR product) encoding the protein of interest. The plasmid must contain a T7 promoter (see page 12).
- Thermomixer (Eppendorf, Hamburg, Germany)

### 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.
- EasyXpress Insect Extract is provided as 4 individual aliquots in single tubes. Once thawed, store EasyXpress Insect extract on ice and use within 4 hours. Do not refreeze and thaw more than four times. Refreeze the extracts in liquid nitrogen.
- The recommended incubation temperature for transcription is 37°C, the incubation temperature for protein synthesis is 27°C.
- We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at –20°C.

### Procedure

#### In vitro transcription reaction

1. **Thaw and store 5x Transcription Buffer, 5x NTP Mix, 20x Enzyme Mix, RNase-Free Water, and EasyXpress Insect Positive Control DNA on ice.**  
Before use, gently vortex and briefly centrifuge each tube to ensure homogeneity of solutions.
2. **Pipet together the components of the three transcription reactions shown in Table 6 in two DNase- and RNase-free 1.5 ml microcentrifuge tubes at room temperature (15–25°C). Label each tube clearly.**

It is important to add the reaction components in the order shown in Table 6.

**Table 6. Pipetting scheme for transcription reactions**

Reagent	Target protein template reaction	Positive control reaction	No template control reaction
RNase-Free water	157.5 µl	8.75 µl	13.75 µl
5x Transcription Buffer	90 µl	5 µl	5 µl
5x NTP Mix	90 µl	5 µl	5 µl
20x Enzyme Mix	22.5 µl	1.25 µl	1.25 µl
EasyXpress Insect Positive Control DNA	—	5 µl	—
DNA	90 µl*	—	—
<b>Total</b>	<b>450 µl</b>	<b>25 µl</b>	<b>25 µl</b>

\* For **linearized DNA encoding mRNAs of less than 1500 bases**, 18 µg linearized DNA template should be added to each 450 µl in vitro transcription reaction at a concentration of 0.2 µg/µl.

For **linearized DNA encoding mRNAs of greater than 1500 bases**, 27 µg linearized DNA template should be added to each 450 µl in vitro transcription reaction at a concentration of 0.3 µg/µl.

For **circular plasmid DNA**, 27 µg template should be added to each 450 µl reaction.

For **PCR products**, 9 µg template should be added to each 450 µl reaction.

3. Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.
4. Incubate the in vitro transcription reactions for 120 min at 37°C.
5. Centrifuge the reactions for 1 min at 12,000 x g at room temperature (15–25°C). Pipet the reaction supernatants into clean DNase- and RNase-free 1.5 ml microcentrifuge tubes and keep at room temperature (15–25°C). Label each tube clearly.

We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at –20°C.

### In vitro translation reaction

In this part of the protocol, mRNA is cleaned up using DyeEx gel-filtration spin columns before being pooled and added to the translation reaction.

### Important note before starting

All centrifugation steps are performed at 750 x g in a conventional microcentrifuge. The appropriate speed for individual centrifuges can be calculated as follows:  
 $\text{rpm} = 1000 \times \sqrt{(750/1.12 \text{ r})}$  (r = radius of rotor in mm).



**Table 7. Examples of suitable microcentrifuges**

<b>Microcentrifuge</b>	<b>Speed corresponding to 750 x g</b>
Eppendorf® Centrifuge 5415C	3000 rpm
Eppendorf Centrifuge 5417C	2700 rpm
Heraeus Biofuge 15	2800 rpm
Hettich Mikro 24-48	2630 rpm
Beckman GS15R	2100 rpm
Hettich Mikro EBA12	2700 rpm

**Procedure**

- 6. Gently vortex 20 DyeEx spin columns to resuspend the resin.**
- 7. Loosen the caps of the columns a quarter turn.**  
This is necessary to avoid a vacuum developing inside the spin column.
- 8. Snap off the bottom closure of the spin columns (Figure 10), and place the spin columns into a DNase- and RNase-free 2 ml microcentrifuge tube.**



**Figure 10. Snapping off the bottom closure of the DyeEx 2.0 spin column (do not screw).**

9. **Centrifuge for 3 min at the calculated speed (see Table 7).**
10. **Carefully transfer the spin columns to clean centrifuge tubes. Slowly apply 20  $\mu$ l in vitro transcription reaction to the gel bed (Figure 11) of each spin column.**

Pipet the in vitro transcription reaction directly onto the center of the slanted gel-bed surface (Figure 11). Do not allow the reaction mixture or the pipet tip to touch the sides of the column.

The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip. It is not necessary to replace the lid on the column.

11. **Centrifuge for 3 min at the calculated speed.**
12. **Remove the spin columns from their microcentrifuge tubes. Pool and keep the eluates at room temperature and proceed immediately with the in vitro translation reaction.**

The eluates contain the purified RNA.



Figure 11. Instructions for sample application to the DyeEx 2.0 spin column.

13. **Thaw and store EasyXpress Insect Reaction Buffer, EasyXpress Insect Extract, EasyXpress Energy Mix, and RNase-Free Water on ice.**
14. **Pipet together the components of the three translation reactions shown in Table 8 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes. Label each tube clearly.**

It is important to add the reaction components in the order shown in Table 8  
Note: If template mRNA has been stored overnight at  $-20^{\circ}\text{C}$ , thaw at room temperature ( $15\text{--}25^{\circ}\text{C}$ ) and vortex briefly before adding to the translation reaction.

**Table 8. Pipetting scheme for high-yield in vitro translation reactions**

<b>Reagent</b>	<b>Target protein synthesis reaction</b>	<b>Positive control reaction</b>	<b>No template control reaction</b>
RNase-Free Water	144 $\mu$ l	8 $\mu$ l	8 $\mu$ l
EasyXpress Insect Reaction Buffer	90 $\mu$ l	5 $\mu$ l	5 $\mu$ l
EasyXpress Insect Extract	360 $\mu$ l	20 $\mu$ l	20 $\mu$ l
Template mRNA *	216 $\mu$ l	12 $\mu$ l	12 $\mu$ l
Insect EasyXpress Energy Mix	90 $\mu$ l	5 $\mu$ l	5 $\mu$ l
<b>Total</b>	<b>900 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>

\* From step 12. Use an aliquot of the DyeEx spin column flow-through from the respective transcription reaction.

- 15. Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
- 16. Incubate the in vitro translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.**
- 17. Proceed with protein analysis or purification.**

## Appendix C: Analyzing the Luciferase Positive-Control Translation Reaction

The positive-control reaction is performed using a DNA template that encodes a 6xHis-tagged luciferase. Luciferase is a monomeric protein with a molecular weight of 61 kDa, which is found in the cells of bioluminescent organisms and catalyzes the oxidation of luciferin and ATP, producing light. Only full-length luciferase is active.

For a luciferase activity assay using a commercially available kit (e.g., Promega Luciferase Assay Reagent, cat. no. E1483), use 10  $\mu$ l crude luciferase translation reaction.

For western blot analysis, load 2  $\mu$ l (chemiluminescent detection) or 8  $\mu$ l (chromogenic detection) crude luciferase translation reaction onto a 12% SDS-PAGE gel, transfer the protein from the gel onto nitrocellulose, and detect the luciferase protein with an anti-His-tag antibody (e.g., Penta-His Antibody, cat. no. 34660) or an anti-luciferase antibody (e.g., Monoclonal Anti-Luciferase Antibody, Sigma, cat. no. L2164).

For quantification of expressed luciferase using incorporation of radioactively-labeled amino acids, see Appendix D.

## Appendix D: Incorporating Radioactive Labels into Proteins for Quantification

Protein expressed using the EasyXpress Insect Kit II can be quantified by incorporating radioactive amino acids (e.g.,  $^{14}\text{C}$ -leucine or  $^{35}\text{S}$ -methionine).  $^{14}\text{C}$  is more stable than  $^{35}\text{S}$  and its use is recommended for accurate quantification. However,  $^{35}\text{S}$  provides a stronger signal. It is recommended that 1500 pmol  $^{14}\text{C}$ -labeled leucine ( $^{14}\text{C}$ -Leu) is added per 50  $\mu\text{l}$  reaction. A protocol and example calculation of protein yield is given below.

### Materials and equipment to be supplied by user\*

- Linearized plasmid DNA encoding protein of interest
- Thermomixer
- 300  $\mu\text{M}$   $^{14}\text{C}$ -labeled leucine ( $^{14}\text{C}$ -Leu, 100 cpm/pmol)

### Procedure

1. **Perform a transcription reaction (see protocol on page 20).**
2. **Thaw EasyXpress Insect Reaction Buffer (blue screw-cap) on ice. Thaw RNase-free water (colorless screw-cap) and EasyXpress Positive-Control DNA (violet screw-cap) at room temperature (15–25°C).**
3. **For each reaction to be performed, thaw 20  $\mu\text{l}$  of EasyXpress Insect Extract (colorless snap-cap) on ice.**
4. **Set up the three reactions detailed in Table 9.**  
It is important to follow the order of addition given in the table.
5. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
6. **Incubate the in vitro translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.**
7. **Use a 10  $\mu\text{l}$  aliquot of each reaction for quantification of protein synthesis by TCA precipitation (see page 38).**

Alternatively, the reactions can be separated by SDS-PAGE and analyzed by autoradiography after drying the gel.

\* 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.

**Table 9. Pipetting scheme for radioactive labeling reactions**

Reagent	Target protein synthesis reaction	Positive control reaction	No template control reaction
RNase-free water	10 $\mu$ l	10 $\mu$ l	10 $\mu$ l
EasyXpress Insect Reaction Buffer	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
300 $\mu$ M $^{14}$ C-Leu	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
EasyXpress Insect Extract	20 $\mu$ l	20 $\mu$ l	20 $\mu$ l
Template mRNA*	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
EasyXpress Insect Energy Mix	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
<b>Total</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>

\* From in vitro transcription step 5, page 20. Use an aliquot of the respective transcription reaction.

## Labeling with $^{35}$ S-Methionine

As an alternative to  $^{14}$ C-leucine labeling, in vitro translated proteins can be labeled using  $^{35}$ S-methionine ( $^{35}$ S-Met). A pipetting scheme for the reactions is given in the table below. Labeling proteins with  $^{35}$ S-methionine gives stronger signals. We recommend using 1  $\mu$ l of 15  $\mu$ M  $^{35}$ S-methionine (1000 Ci/mmol) for labeling each reaction.

**Table 10. Pipetting scheme for radioactive labeling reactions ( $^{35}$ S)**

Reagent	Target protein synthesis reaction	Positive control reaction	No template control reaction
RNase-free water	14 $\mu$ l	14 $\mu$ l	14 $\mu$ l
EasyXpress Insect Reaction Buffer	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
15 $\mu$ M $^{35}$ S-Met	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l
EasyXpress Insect Extract	20 $\mu$ l	20 $\mu$ l	20 $\mu$ l
Template mRNA*	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
EasyXpress Insect Energy Mix	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
<b>Total</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>	<b>50 <math>\mu</math>l</b>

\* From in vitro transcription step 5, page 20. Use an aliquot of the respective transcription reaction.

# 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 Insect Kit II reactions.

## Materials and equipment to be supplied by the user\*

- 5% (w/v) trichloroacetic acid (TCA) and acetone
- Casein acid hydrolysate (e.g., Sigma, cat. no. A 2427)
- Glass microfiber 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) and scintillation counter

## Procedure

1. **Briefly vortex the in vitro translation reaction mixture and transfer a 10 µl aliquot to a 10 ml test tube.**
2. **Add 3 ml of 5% TCA solution containing 2% (w/v) casein acid 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 on ice for at least 30 min to precipitate the synthesized proteins.**
5. **Collect the precipitate on a glass microfiber 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 aliquots of 5% (w/v) TCA.**
7. **Dry the filter by rinsing it 2 times with 3 ml aliquots 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.**
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 10 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.

\* 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.

## Calculation of protein synthesis yield

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

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

### Example calculations

Template	Luciferase control DNA
Molecular weight	61,710 g/mol
Leu residues	51
Met residues	14

### Labeling with $^{14}\text{C}$ -leucine

Leucine concentration	200 $\mu\text{M}$	unlabeled in the kit
$^{14}\text{C}$ -Leu concentration	30 $\mu\text{M}$	
Total leucine concentration	230 $\mu\text{M}$	13.04 cpm/pmol

### Measured radioactivity

TCA precipitated sample (10 $\mu\text{l}$ )	2100 cpm = 210 cpm/ $\mu\text{l}$
Total radioactivity (10 $\mu\text{l}$ sample)	30,000 cpm = 3000 cpm/ $\mu\text{l}$

$$\text{Percentage of } ^{14}\text{C-Leu incorporated} = \frac{210 \times 100}{3000} = 7\%$$

$$\text{Yield } (\mu\text{g/ml}) = \frac{7\% \times 0.01 \times 230 \mu\text{M} \times 61,710 \text{ g/mol}}{51 \times 1000} = 19.48 \mu\text{g/ml}$$

### Labeling with $^{35}\text{S}$ -methionine

Proteins labeled with  $^{35}\text{S}$ -methionine gives stronger signals than  $^{14}\text{C}$ -labeled proteins. A typical commercially available  $^{35}\text{S}$ -methionine solution has a specific activity of 1  $\mu\text{Ci/pmol} = 2.22 \times 10^6$  cpm/pmol and a concentration of 15  $\mu\text{M}$ . This example is based on the addition of 1  $\mu\text{l}$  of  $^{35}\text{S}$ -methionine solution (= 1  $\mu\text{Ci}$ ) for a 50  $\mu\text{l}$  in vitro translation reaction.

Methionine concentration	200 $\mu\text{M}$	unlabeled in the kit
$^{35}\text{S}$ -Met concentration	0.3 $\mu\text{M}$	$2.22 \times 10^6$ cpm/pmol
Total methionine concentration	200.3 $\mu\text{M}$	3325 cpm/pmol

**Measured radioactivity**

TCA precipitated sample (10 µl)      166,500 cpm = 16,650 cpm/µl

Total radioactivity (10 µl sample)      6,660,000 cpm = 666,000 cpm/µl

$$\text{Percentage of } ^{35}\text{S-Met incorporated} = \frac{16,650 \times 100}{666,000} = 2.5\%$$

$$\text{Yield (}\mu\text{g/ml)} = \frac{2.5\% \times 0.01 \times 200.3 \mu\text{M} \times 61,710 \text{ g/mol}}{14 \times 1000} = 22.07 \mu\text{g/ml}$$



## Appendix E: Cloning Expression Sequences into the pIX4.0 Vector

The pIX4.0 cloning and expression vector has been specially developed to provide high expression rates in insect-cell lysates. It contains the T7 transcription promoter, optimized 5'- and 3'-untranslated regions (UTRs), a multiple cloning site (MCS), the T7 transcription terminator to stabilize mRNA against exonucleolytic digestion, several alternative restriction sites for plasmid linearization with blunt ends for effective run-off transcription, hybridization sequences for sequencing primers (M13 forward, reverse), an ampicillin resistance marker, and a high-copy origin of replication.

### Cloning strategies

<i>NcoI</i>	<i>BseRI</i>	<i>XhoI</i>	<i>NotI</i>	<i>PstI</i>	<i>BglII</i>	<i>SpeI</i>
<b>CCATG</b> GGAGACCCCTCCTCGAGCGGCCGCCTGCAGATCTAAATAATAAGTAATTAAGTAGT						
GGTACCCCTCTGGGAGGAGCTCGCCGGCGGACGTCTAGATTATTATTTCATTAATTGATCA						
Met Gly Asp Pro Ser Ser Gly Arg Leu Gln Ile						

#### The pIX4.0 multiple cloning site

If the *NcoI* cloning site is used, it should be noted that because the G 3' of the ATG start codon is an absolute requirement for *NcoI* restriction, sequences that are cloned into the pIX4.0 vector using the *NcoI* restriction site will start with an N-terminus Met followed by the amino acid Gly, Glu, Asp, Ala, or Val. If the *XhoI*, *NotI*, *PstI*, *BglII*, or *SpeI* cloning sites are used, it should be noted that because translation commences at the ATG start codon, additional amino acids will be added to the N-terminus of the native protein sequence. If native structure proteins, which are free of vector-encoded amino acids, are required, the *BseRI* cloning site should be used.

The cloning options can be summarized as follows:

- Protein sequence starts with an N-Terminus Met followed by the amino acid Gly, Glu, Asp, Ala, or Val. See "Cloning into pIX4.0 via *NcoI*", page 42.
- Proteins are synthesized free of any additional vector amino acids. See "Cloning into pIX4.0 via *BseRI*", page 44.
- Protein contains up to 6 vector-encoded N-terminal amino acids. See "Cloning into pIX4.0 via *XhoI*, *NotI*, *PstI*, *BglII*", page 46.
- Protein is synthesized with an N-terminal 6xHis or *Strep-tag*®. See "PCR-mediated addition of affinity-tag sequences", page 48.
- Protein is synthesized with a C-terminal 6xHis or *Strep-tag*. See "PCR-mediated addition of affinity-tag sequences", page 48.
- Protein contains an intrinsic N-terminal sequence motif (signal sequence/signal peptide) directing the protein to the secretory pathway (e.g., glycoproteins), Appendix F, page 50.

## Cloning into pIX4.0 via *Nco*I

For the PCR, a sense primer with the following structure should be synthesized:



### Sense primer

The restriction enzyme recognition sequence is underlined and the translation start codon ATG is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (M<sub>20</sub>) corresponds to bases 5–24 of the target protein coding sequence.

For the PCR, an antisense primer with the following structure should be synthesized:

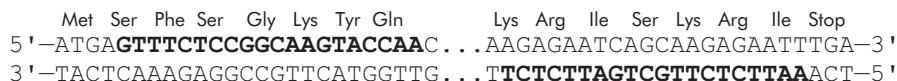


### Antisense primer

The restriction enzyme recognition sequence is underlined and the translation stop is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (N<sub>20</sub>) corresponds to the antisense sequence of the last 20 bases of the target protein coding sequence.

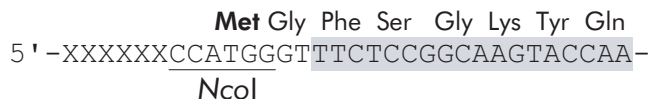
## Example: Primer design for cloning of FABP using the *Nco*I and *Bgl*II cloning sites

The beginning and ends of the protein coding sequence for fatty acid binding protein (FABP) are shown below. The bases that will serve as PCR primer binding sites (bases 5–24 [sense] and the last 20 bases [antisense]) are in bold.



### Coding sequence of FABP

For the PCR the following sense primer is constructed. Native target protein sequence is shaded.



### Sense primer

It should be noted that due to the presence of the G 3' of the ATG start codon, the Ser residue in the native sequence will be converted to a Gly.

6For the PCR the following antisense primer is constructed. Native target protein sequence is shaded.

**Stop** Ile Arg Lys Ser Ile Arg  
 5' -XXXXXXAGATCTTTAAATTCTCTTGCTGATTCTCT-3'  
                     Bg/II

Antisense primer

As a first stage of the cloning procedure, the PCR product is digested with NcoI. Native target protein sequence is shaded.

↓  
 5' -XXXXXXCCATGGGTTTCTCCGGCAAGTACCAA-3'  
 3' -XXXXXXGGTACCCAAGAGGCCGTTTCATGGTT-5'  
                     NcoI ↑



5' -CATGGGTTTCTCCGGCAAGTACCAA... 3'  
 3' -          CCAAAGAGGCCGTTTCATGGTT... 5'

The vector is then digested with NcoI.

↓  
 5' UTR. CCATGGGGAGACCCCTCCTCGAGCAGTTTCTCCGGCAAGTACCA...  
           GGTACCCTCTGGGGAGGAGCTCGTCAAAGAGGCCGTTTCATGGT  
           NcoI ↑



5' UTR. C-3'  
           GGTAC-5'

The digested PCR product and vector are then ligated. Native target protein sequence is shaded. Vector-encoded amino acids are underlined.

Met Gly Ser Phe Ser Gly Lys Tyr  
 5' UTR... CCATGGGGTTTCTCCGGCAAGTACCA...  
           GGTACCCTAAAGAGGCCGTTTCATGGT...

## Cloning into pIX4.0 via *Bse*RI

For the PCR, a sense primer with the following structure should be synthesized:

5' -XXXXXXGAGGAGGTCTCCC**ATG** (M<sub>20</sub>) -3'  
                                  *Bse*RI                                  Met

### Sense primer

The restriction enzyme recognition sequence is underlined and the translation start codon ATG is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (M<sub>20</sub>) corresponds to bases 4–23 of the target protein coding sequence.

For the PCR, an antisense primer with the following structure should be synthesized:

5' -XXXXXXAGATCT**TTA** (N<sub>20</sub>) -3'  
                                  *Bg*/III    Stop

### Antisense primer

The restriction enzyme recognition sequence is underlined and the translation stop is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (N<sub>20</sub>) corresponds to the antisense sequence of the last 20 bases of the target protein coding sequence.

A section of the resulting double-stranded PCR product is shown below. It should be noted that *Bse*RI cuts at a staggered site 10 and 8 bases away from its recognition site (arrowed). The recognition site is underlined and the ATG start codon appears in bold.

↓

5' XXXXXXGAGGAGGTCTCCC**ATG**NNN . . . 3'  
      XXXXXXCTCCTCCAGAGGGTACNNN . . . 5'  
                                  *Bse*RI                                  ↑

### PCR product sequence

A section of the pIX4.0 vector MCS is shown below. As before, *Bse*RI cuts at a staggered site 10 and 8 bases away from its recognition site (arrowed). In this case the recognition site is located downstream of the ATG start codon (bold). The recognition site is underlined.

↓

. . . CC**ATG**GGAGACCCCTCCTC . . .  
      . . . GGTACCCTCTGGGGAGGAG . . .  
                                  ↑                                  *Bse*RI

### pIX4.0 vector sequence

**Example: Primer design for cloning of FABP using the *Bse*RI and *Bgl*II cloning sites**

The beginning and ends of the protein coding sequence for fatty acid binding protein (FABP) are shown below. The bases that will serve as PCR primer binding sites (bases 4–23 [sense] and the last 20 bases [antisense]) are in bold.

Met Ser Phe Ser Gly Lys Tyr Gln Lys Arg Ile Ser Lys Arg Ile Stop  
5'–ATG**AGTTTCTCCGGCAAGTACCA**AC...AAGAGAATCAGCAAGAGAATTGA–3'  
3'–TACTCAAAGAGGCCGTTTCATGGTTG...T**TCTCTTAGTCGTTCTCTTAA**ACT–5'

**Coding sequence of FABP**

For the PCR the following primers are constructed. Native target protein sequence is shaded.

Met Ser Phe Ser Gly Lys Tyr Gln  
5'–XXXXXXGAGGAGGTCTCCC**ATGAGTTTCTCCGGCAAGTACCA**–3'  
BseRI

Sense primer

Stop Ile Arg Lys Ser Ile Arg  
5'–XXXXXXAGATCT**TTA**AATTCTCTTGCTGATTCTCT–3'  
BglII

Antisense primer

As a first stage of the cloning procedure, the PCR product is digested with *Bse*RI. Native target protein sequence is shaded.

↓  
5'–XXXXXXGAGGAGGTCTCCC**ATGAGTTTCTCCGGCAAGTACCA**...3'  
3'–XXXXXXCTCCTCCAGAGGGTACTCAAAGAGGCCGTTTCATGGT...5'  
BseRI ↑

↓  
5'–**AGTTTCTCCGGCAAGTACCA**...3'  
3'–**ACTCAAAGAGGCCGTTTCATGGT**...5'

Promoter-5'UTR-ATCC**AT**GGGAGACCCCTCCTC...  
 .....TAGGTACCCTCTGGGGAGGAG...  
 ↑ BseRI

The digested PCR product and vector are then ligated. Native target protein sequence is shaded.

### Cloning into pIX4.0 via *Xho*I, *Not*I, *Pst*I, or *Bgl*II

Sequences that are cloned into the pX4.0 vector using the *Xho*I, *Not*I, *Pst*I, or *Bgl*II restriction sites will contain up to six vector encoded amino acids (see below). Suggested PCR primers for cloning are given below. Restriction sites are underlined and the translation start codon is in bold. X can be any base, the presence of this short sequence increases digestion efficiency. (M<sub>20</sub>) corresponds to bases 4–23 of the target protein coding sequence. (N<sub>20</sub>) corresponds to the final 20 coding bases of the target protein coding sequence.

### Cloning into pIX4.0 via *Xho*I

For the PCR, a sense primer with the following structure should be synthesized:

5'-XXXXXXCTCGAGC (M<sub>20</sub>)-3'

**Sense primer**

The restriction enzyme recognition sequence is underlined. X can be any base; the presence of this short sequence increases digestion efficiency. (M<sub>20</sub>) corresponds to bases 4–23 of the target protein coding sequence. The base C between the *Xho*I recognition sequence and the protein coding sequence is required to ensure that the coding sequence codons are shifted into frame.

### Example: Primer design for cloning of FABP using the *Xho*I and *Bgl*II cloning sites

The beginning and ends of the protein coding sequence for fatty acid binding protein (FABP) are shown below. The bases that will serve as PCR primer binding sites (bases 4–23 [sense] and the last 20 bases [antisense]) are in bold.

Met Ser Phe Ser Gly Lys Tyr Gln Lys Arg Ile Ser Lys Arg Ile Stop  
5'–ATG**AGTTTCTCCGGCAAGTACCA**AC...AAGAGAATCAGCAAGAGAATTTGA–3'  
3'–TACTCAAAGAGGCCGTTTCATGGTTG...T**TCTCTTAGTCGTTTCTCTTAA**ACT–5'

#### Coding sequence of FABP

For the PCR the following primers are constructed. Native target protein sequence is shaded.

Ser Ser Ser Phe Ser Gly Lys Tyr  
5'–XXXXXXCTCGAGC**AGTTTCTCCGGCAAGTACCA**–3'  
XhoI

Sense primer

Stop Ile Arg Lys Ser Ile Arg  
5'–XXXXXXAGATCT**TTA**AATTCTCTTGCTGATTCTCT–3'  
BglII

Antisense primer

As a first stage of the cloning procedure, the PCR product is digested with *Xho*I. Native target protein sequence is shaded.

↓  
5'–XXXXXXCTCGAGC**AGTTTCTCCGGCAAGTACCA**...3  
3'–XXXXXXGAGCTCGTCAAAGAGGCCGTTTCATGGT...5'  
XhoI ↑



5'–TCGAGC**AGTTTCTCCGGCAAGTACCA**...3'  
3'–CGTCAAAGAGGCCGTTTCATGGT...5'

The vector is then digested with *Xho*I.

↓

Met Gly Asp Pro Ser

5' UTR. CC**ATG**GGAGACCCCTCCTCGAGCAGTTTCTCCGGCAAGTACCA...  
 GGTACCCTCTGGGGAGGAGCTCGTCAAAGAGGCCGTTTCATGGT

XhoI ↑

↓

5' UTR. CC**ATG**GGAGACCCCTCC-3'  
 GGTACCCTCTGGGGAGGAGCT-5'

The digested PCR product and vector are then ligated. Native target protein sequence is shaded. Vector-encoded amino acids are underlined.

Met Gly Asp Pro Ser Ser Ser Ser Phe Ser Gly Lys Tyr

5' UTR. ... CC**ATG**GGAGACCCCTCCTCGAGCAGTTTCTCCGGCAAGTACCA  
 GGTACCCTCTGGGGAGGAGCTCGTCAAAGAGGCCGTTTCATGGT

### PCR-mediated addition of affinity-tag sequences

The PCR primers listed below can be used to add affinity-tag coding sequences to expression constructs. Start codons are in bold, and restriction enzyme recognition sites are underlined. If the target protein coding bases lead to the formation of mRNA secondary structures, altering the amino acid codons may improve results.

### Sense primers for addition of N-terminal affinity tags

#### Using the *NcoI* cloning site

N-terminal *Strep*-tag

Met Asp Trp Ser His Pro Gln Phe Glu Lys Ser Ala

5' -XXXXXXCC**ATG**GATTGGTCTCATCCGCAATTCGAAAAAAGCGCT (N<sub>20</sub>) -3'

N-terminal 6xHis tag

Met Asp His His His His His His

5' -XXXXXXCC**ATG**GATCATCATCACCATCACCAC (N<sub>20</sub>) -3'



## Using the *Bse*RI cloning site

N-terminal *Strep*-tag

Met Trp Ser His Pro Gln Phe Glu Lys Ser Ala  
XXXXXXGAGGAGGTCTCCC**ATG**TGGTCTCATCCGCAATTCGAAAAAAGCGCT (N<sub>20</sub>)

N-terminal 6xHis tag

Met His His His His His His  
5' XXXXXXGAGGAGGTCTCCC**ATG**CATCATCACCATCACCAC (N<sub>20</sub>) – 3'

## Using the *Xho*I cloning site

N-terminal *Strep*-tag

Ser Ser Trp Ser His Pro Gln Phe Glu Lys Ser Ala  
5' –XXXXXXCTCGAGCTGGTCTCATCCGCAATTCGAAAAAAGCGCT (N<sub>20</sub>) – 3'

N-terminal 6xHis tag

Ser Ser His His His His His  
5' –XXXXXXCTCGAGCCATCATCACCATCACCAC (N<sub>20</sub>) – 3'

## Antisense primers for addition of C-terminal affinity tags

### Using the *Bgl*II cloning site

C-terminal *Strep*-tag

Stop Lys Glu Phe Gln Pro His Ser Trp Ala Ser  
5' –XXXXXXAGATCT**TTA**TTTTTCGAATTGCGGATGAGACCAAGCGCT (N<sub>20</sub>) – 3'

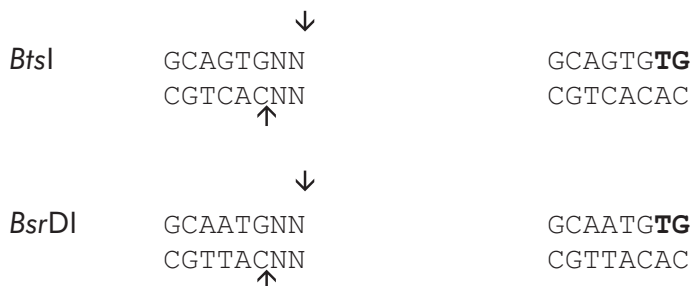
C-terminal 6xHis tag

Stop His His His His His His  
5' –XXXXXXAGATCT**TTA**GTGGTGATGGTGATGATG (N<sub>20</sub>) – 3'

## Additional cloning options for pIX4.0

If the recognition sequence for *Nco*I is present in the target protein's coding sequence, or the codon for the N-terminal amino acid cannot commence with a guanine, *Nco*I cannot be used for cloning. In such cases, the recognition sequence for *Bsp*HI (TCATGA) or *Pci*I (ACATGT) can be added to the target gene sequence by PCR; of course these sequences should not appear in the target protein coding sequence. The *Bsp*HI or *Pci*I restricted insert can be ligated into the *Nco*I restricted vector. Using *Bsp*HI or *Pci*I for restriction dictates that the first base of the N-terminal codon is A or T respectively.

If the recognition sequence for *Bse*RI is present in the target protein's coding sequence, *Bse*RI cannot be used for cloning. In such cases, the recognition sequence for *Bts*I or *Bsr*DI (see below) can be added to the target gene sequence by PCR; of course these sequences should not appear in the target protein coding sequence.



The *Bts*I or *Bsr*DI restricted insert can be ligated into the *Bse*RI restricted vector if the DNA sense strand contains TG at the position indicated above in bold. The TG motif forms the second and third position of the ATG start codon. Additional restriction enzymes that can be used for restriction of PCR products that can be ligated into a *Bse*RI restricted vector are *Bce*AI, *Bpm*I, *Bpu*EI, *Bsg*I, *Ec*II, or *Mme*I.

## Appendix F: Synthesis of Secreted Proteins and Glycoproteins Using the EasyXpress Insect Kit II

In eukaryotic cells, glycosylated proteins and proteins that are destined for excretion are synthesized by ribosomes associated with the membranes of the endoplasmic reticulum (ER). These classes of proteins are synthesized with an N-terminus containing a so-called signal sequence or signal peptide. The signal peptide usually consists of 13–36 predominantly hydrophobic residues. As the protein is synthesized, the signal sequence is passed through the ER membrane into the lumen of the ER. After the growing protein chain has reached a certain length, the signal peptide is removed by the action of signal peptidases. Protein synthesis continues, and if the protein will be secreted, it ends up completely in the lumen of the ER. In mammalian cells, glycan group attachment to glycoproteins via N-glycosidic linkages occurs predominantly in the lumen of the ER.

The cell lysate in the EasyXpress Insect Kit II contains microsomal membranes derived from the endoplasmic reticulum of insect cells. To ensure efficient translocation of a glycoprotein or a secreted protein across the membrane of these microsomes, a signal peptide must be present at the N-terminus of the *in vitro* synthesized protein. In such cases, no N-terminal tag sequence should be added to the protein. Addition of C-terminal affinity tags is possible.

Determining the presence of a signal peptide in the protein of interest is an important first step prior to its in vitro synthesis. Internet-accessible tools (e.g., SignalP; <http://www.cbs.dtu.dk/services/SignalP/>) are capable of predicting the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms. These native signal sequences differ widely in their ability to facilitate protein translocation. Moreover, foreign signal sequences (e.g., mammalian sequences) may be less efficient in insect cell lysates. Therefore, substitution of the foreign protein's own signal sequence for a powerful insect melittin signal sequence (see Figure XX) often results in more efficient protein translocation and glycosylation.

PCR-mediated substitution of the N-terminal signal sequence and cloning of the amplification product into the expression vector pIX4.0 can significantly improve the results of cell-free expression reactions using the EasyXpress Insect Kit II.

**M K F L V N V A L V F M V V Y I S Y I Y A\* D**  
 ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTATGGTCGTATACATTTCTTACATCTATGCGGAC

\* Position where the signal peptide cleavage occurs.

**Figure 11. Coding and amino acid sequence of the melittin signal peptide.**

We recommend inserting this signal peptide coding sequence by generating a sequence-verified synthetic gene, flanked by *Nco*I and *Xho*I sites, and cloning it into *Nco*I/*Xho*I digested pIX4.0 (→ pIX4.0/melittin). Subsequently, protein coding sequences (lacking endogenous signal sequences) should be amplified by PCR using primers coding for flanking *Xho*I/*Bgl*II or *Xho*I/*Spe*I restriction sites and cloned into the *Xho*I/*Bgl*II or *Xho*I/*Spe*I digested pIX4.0/melittin vector.

## Ordering Information

Product	Contents	Cat. no.
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 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 pIX4.0 Vector	25 µg vector DNA for efficient synthesis of proteins using the EasyXpress Insect Kit II	32713
EasyXpress Random Biotin Kit	For 60 x 50 µl reactions: biotinyl-lysyl tRNA (Phe)	32612
<b>Related products</b>		
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 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 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 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 Kit (5)	For 5 x 50 µl reactions: <i>E. coli</i> extract, reaction buffer, RNase-free Water, and positive-control DNA	32501

## Ordering Information

Product	Contents	Cat. no.
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 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 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
EasyXpress NMR Protein Synthesis Kit – A	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Ala (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32530
EasyXpress NMR Protein Synthesis Kit – N	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Asn (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32531
EasyXpress NMR Protein Synthesis Kit – D	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Asp (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32532
EasyXpress NMR Protein Synthesis Kit – C	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Cys (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32533

## Ordering Information

Product	Contents	Cat. no.
EasyXpress NMR Protein Synthesis Kit – E	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Glu (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32534
EasyXpress NMR Protein Synthesis Kit – Q	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Gln (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32535
EasyXpress NMR Protein Synthesis Kit – G	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Gly (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32536
EasyXpress NMR Protein Synthesis Kit – H	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o His (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32537
EasyXpress NMR Protein Synthesis Kit – I	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Ile (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32538
EasyXpress NMR Protein Synthesis Kit – L	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Leu (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32539
EasyXpress NMR Protein Synthesis Kit – M	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Met (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32540

## Ordering Information

Product	Contents	Cat. no.
EasyXpress NMR Protein Synthesis Kit – F	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Phe (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32541
EasyXpress NMR Protein Synthesis Kit – P	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Pro (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32542
EasyXpress NMR Protein Synthesis Kit – W	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Trp (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32543
EasyXpress NMR Protein Synthesis Kit – Y	For 2 x 5 ml reactions: <i>E. coli</i> extract, reaction buffers, amino acid mix w/o Tyr (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	32544
<b>Protein purification</b>		
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
<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

\* Larger pack sizes available; please inquire.

## Ordering Information

Product	Contents	Cat. no.
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
<b>Protein detection</b>		
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 µg)	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
<b>Plasmid DNA purification</b>		
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

\* Larger pack sizes available; please inquire.



# Ordering Information

Product	Contents	Cat. no.
<b>Linearized plasmid DNA purification</b>		
QIAquick Nucleotide Removal Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28304

\* Larger sizes available; please inquire

## Notes

Trademarks: QIAGEN®, QIAprep®, QIAquick®, DyeEx®, EasyXpress®, HiSpeed® (QIAGEN Group); Eppendorf®, Thermomixer®, (Eppendorf-Netheler-Hinz GmbH); PhosphorImager® (Molecular Dynamics); Ready Protein+™ (Beckman Coulter Inc.); Tween® (ICI Americas Inc.); Whatman® (Whatman PLC)

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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.

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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 1 (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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