April 2008

EasyXpress® Linear Template Kit Plus Handbook

For generation of linear expression templates by PCR and easy cloning of PCR products into the pIX3.0 Vector

EasyXpress Linear Template Kit Plus

EasyXpress pIX3.0 Vector

QIAGEN®

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QIAGEN Sample and Assay Technologies

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QIAGEN sets standards in:

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Contents

Kit Contents

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

EasyXpress pIX3.0 Vector	**Cat. no. 32733**
EasyXpress pIX3.0 Vector (white screw-cap)	25 µg (0.5 µg/µl)
Handbook	1

Storage

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

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

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

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).

Technical Assistance

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

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

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/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

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

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

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

Proteins produced by cell-free expression can be used for the same wide variety of downstream applications as in-vivo produced proteins, including activity assays, structural and functional analyses, protein–protein interaction studies, and the expression and analysis of open reading frames.

The EasyXpress System

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

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

Identification of optimal constructs in a single day

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

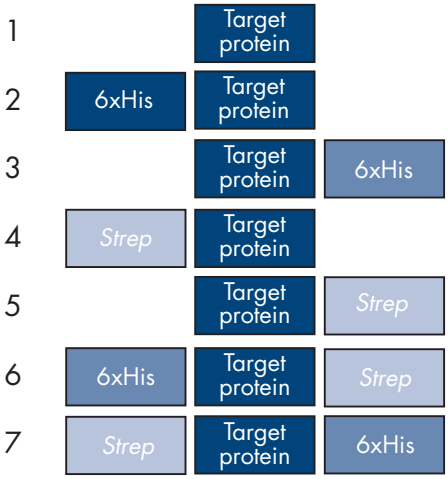


Figure 1. The variety of constructs whose expression can be tested using the Qproteome Linear Template and Protein Synthesis Kits.

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

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

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

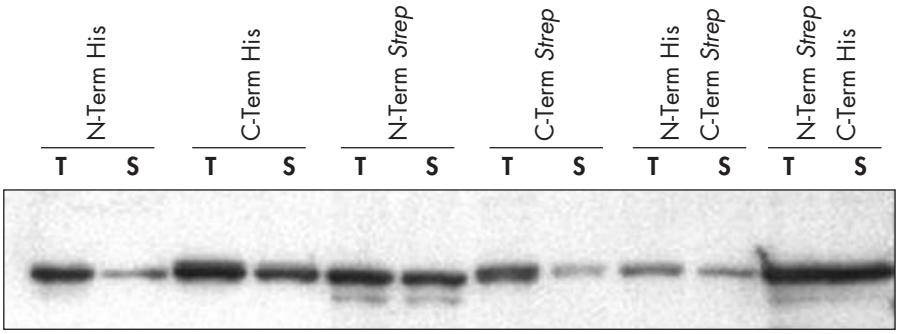


Figure 2. Expression analysis reveals the optimal construct. PCR products expressing IRAK4 and carrying the indicated tag(s) were added to small-scale *E. coli* IVT reactions. Samples of total protein (T, complete reaction) and soluble protein (S, supernatant of complete reaction after centrifugation)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

Scaling up cell-free protein synthesis reactions

Once the optimal construct has been identified it can be directly cloned into the pIX3.0 vector for scaled up in vivo or in vitro expression in *E. coli*. For structural biologists who want to express large amounts of protein containing isotopically labeled or modified amino acids, QIAGEN offers the EasyXpress NMR Protein Synthesis Kit (cat. no. 32526) and EasyXpress Protein Synthesis Mega Kit (cat. no. 32516), which enable up to 5 mg cotranslationally labeled protein to be synthesized and purified in just 5 hours.

To learn more about the EasyXpress system, visit www.qiagen.com/easyxpress or see references 1–2.

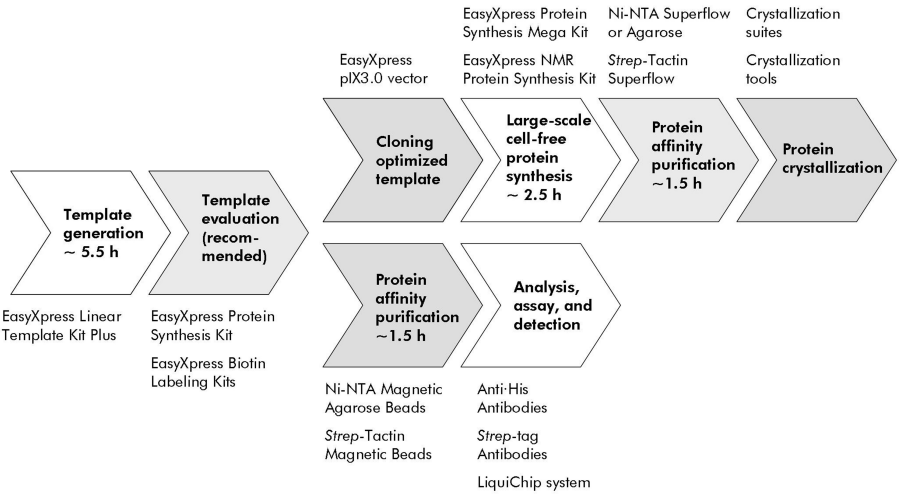


Figure 3. Expression, purification, detection, and analysis form an integrated workflow chain.

Expression testing of QIAgenes Expression Constructs

QIAgenes are available from QIAGEN as genome-wide expression-ready vectors. These are optimized synthetic human genes, ready-cloned into expression vectors covering over 35,000 human ORFs and are now available from the GeneGlobe database (www.geneglobe.com). 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. Initial expression testing of QIAgenes Expression Constructs optimized for *E. coli* expression can be very quickly performed using the EasyXpress Protein Synthesis Kit (5) (cat. no. 32501).

Principle and Procedure

The EasyXpress Linear Template Kit Plus uses a two-step PCR process to generate linear DNA templates for in vitro translation systems. Using specially designed primers, coding DNA sequence is both amplified and supplemented with regulatory elements required for optimal transcription and translation in cell-free expression systems. Specially designed 5' untranslated regions (UTRs) on the sense adapter primer sequences reduce the formation of secondary structure in the translation initiation region, one of the commonest causes of low expression rates. A His-or *Strep*-tag II can be added to either terminus, greatly simplifying protein purification and detection. The N-terminal *Strep*-tag construct also contains a Factor Xa Protease cleavage site, for easy tag removal. The fast procedure enables researchers to discover the optimal template structure within a single working day (Figure 4).

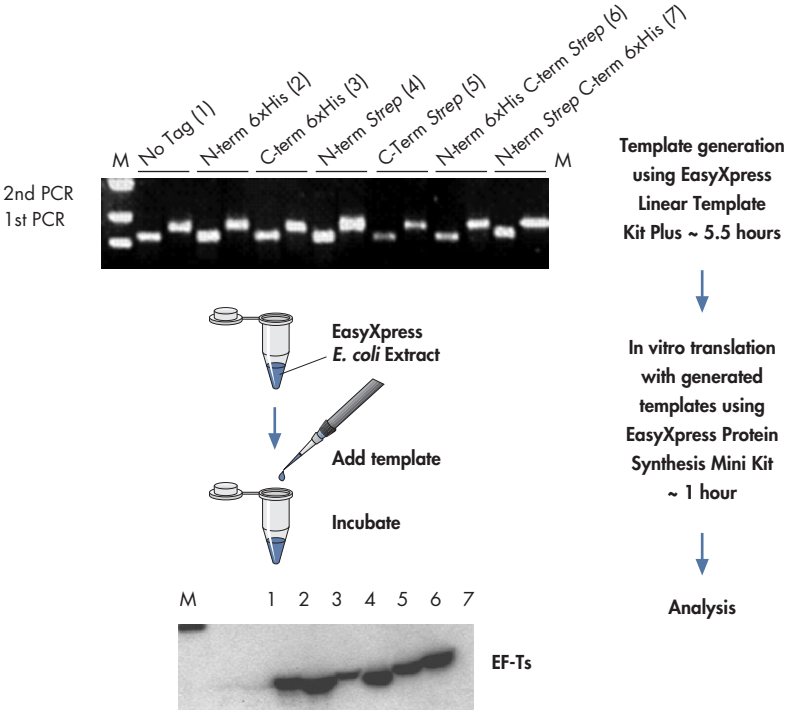


Figure 4. Identification of the optimal expression construct maximizes yields in large-scale in vitro reactions or in vivo expression. The western blot was probed with anti-His- and *Strep*-tag antibodies. Therefore, the untagged protein is not detected.

The EasyXpress pIX3.0 Vector (see Appendix) has been developed to enable easy cloning of PCR products generated using the EasyXpress Linear Template Kit Plus. Its multiple cloning site (MCS) is compatible with restriction sites in the sense and antisense adapter primers supplied with the EasyXpress Linear Template Kit Plus (see Figure 6, page 28). Once cloned into pIX3.0, expression constructs can be used to generate larger amounts of protein in large-scale protein synthesis reactions. Alternatively, the vector can be used to transform *E. coli* cells for conventional in vivo expression.

Generating PCR Products for Use in In Vitro Translation Reactions

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

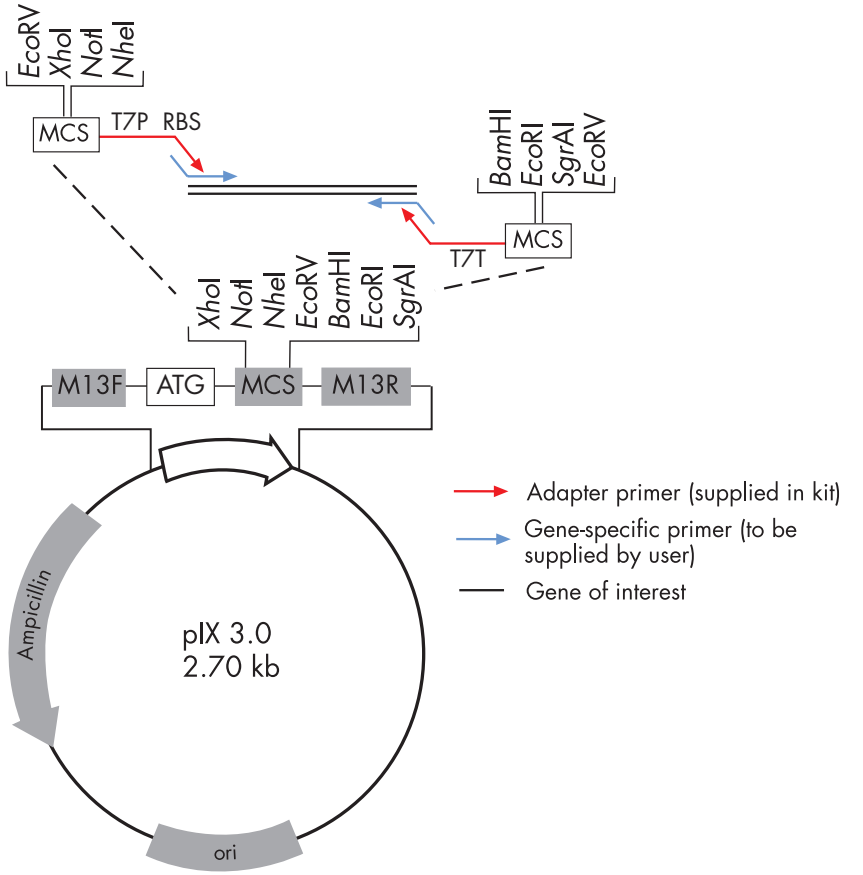


Figure 5 Straightforward cloning into vector pIX3.0 for expression scale-up. T7P: T7promoter; **RBS**: Ribosome binding site; **T7T**: T7 terminator; **MCS**: multiple cloning site.

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

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

Plasmid DNA, genomic DNA mixtures, or cDNA mixtures can be used as a template for the first PCR. Alternatively, cDNA can be generated by reverse transcription PCR (RT-PCR) using a gene-specific antisense primer (with defined 5' tail sequence, see Table 1, page 16), total RNA, and a reverse transcriptase. QIAGEN offers Omniscript® and Sensiscript® RT Kits for efficient RT-PCR. Rules for the design of the gene-specific PCR primers containing the relevant 5' tails are given on page 15.

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

Strategy for designing gene-specific primers

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

The final PCR product added to the in vitro translation reaction is generated by a two-step PCR procedure. In the first PCR, primers must be designed that are not only specific for the protein of interest, but also provide 5' tails that will act as hybridization sites for adapter primers used in the second PCR. The first step in designing primers is to decide whether an affinity tag should be attached to the protein and at which terminus. Use the 5'-end sequence information in Table 1 and the information below to design forward (sense) and reverse (antisense) primers for protein constructs with an affinity tag at the respective terminus.

Length

The length of the gene-specific sequence should be 17–20 bases (see Table 1). 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.

Table 1. Sequences of Gene-Specific Primers Required for First-Round PCR.

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

Table 2. Sense and Antisense Primer Pairs Required for Second-Round PCR.

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

* Nucleotide sequences of primers can be found in the Appendix on page 26.

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

Positive control for the two-step PCR procedure

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

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

This protocol is made up of two separate PCR procedures. In the first PCR, protein-specific sequence is used as a template. The primers used in this first PCR add sequences that will serve as hybridization sites in a second round of PCR (see Figure 5, page 13). In the second PCR, adapter primers (see Table 2) are used to add sequence that encodes regulatory elements required for efficient expression and optional affinity tags.

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 µl‡	● 1.25 units, ▲ 2.5 units
RNase-Free Water	variable	—
Template DNA		
Template DNA, added in step 4	variable	50–500 ng genomic DNA 1–100 ng cDNA§ 0.5–5.0 ng plasmid DNA
Total volume	**25 µl**	**—**

* contains 15 mM $MgSO_4$

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

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

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

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

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

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

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

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

	Time	Temp.	Comments
Initial activation step	5 min	95°C	ProofStart DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation	1 min	94°C	
Annealing	1 min	50°C	Approximately 5°C below 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

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 4.**
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 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 4	1–2 µl product from first PCR (~100 ng)	
Total volume	**25 µl**	**—**

* Contains 15 mM $MgSO_4$

† For possible adapter primer combinations see Table 2, page 17.

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

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

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

	Time	Temp.	Comments
Initial activation step	5 min	95°C	ProofStart DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation	1 min	94°C	
Annealing	1 min	50°C	Approximately 5°C below 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	

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

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

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

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

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

   0.7 µg DNA (~7 µl of the second PCR) is required for a 50 µl in vitro translation reaction using EasyXpress *E. coli*-based Kits.

   0.5 µg DNA (~ 5 µl of the second PCR) is required for a 25 µl in vitro transcription reaction using the EasyXpress Insect Kit II.

Troubleshooting Guide

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

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

		Comments and suggestions
j)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM $MgSO_4$.
k)	Primer design not optimal	Review primer design, see page 15.
l)	Incorrect dNTP concentration	Ensure that a dNTP concentration of 300 µM of each dNTP was used. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.
Product is multi-banded		
a)	PCR cycling conditions not optimal	Review primer design, see page 15.
b)	Enzyme concentration too low	For PCR fragments <2 kb, use 0.5 µl ProofStart DNA Polymerase per 25 µl reaction. For PCR fragments >2 kb, use 1 µl of enzyme per 25 µl reaction.
c)	Extension time too short	Increase extension time in increments of 1 min. Ensure that an extension time of 2 min per kb DNA was used for fragments >2 kb.
d)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM using the 25 mM $MgSO_4$ solution provided (see *ProofStart PCR Handbook*).
e)	Primer concentration not optimal or primers degraded	For calculation of primer concentration, refer to the Appendix in the *ProofStart PCR Handbook*. Particularly when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.
f)	Primer design not optimal	Review primer design, see page 15.
g)	Incorrect dNTP concentration	Ensure that a dNTP concentration of 300 µM of each dNTP was used. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.

		Comments and suggestions
Product is smeared		
a)	Insufficient starting template	Increase amount of starting template used in PCR.
b)	Extension time too short	Increase extension time in increments of 1 min. Ensure that an extension time of 2 min per kb DNA was used for fragments >2 kb.
c)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM using the 25 mM $MgSO_4$ solution provided (see *ProofStart PCR Handbook*).
d)	dNTP concentration not optimal	Ensure that a dNTP concentration of 300 µM of each dNTP was used. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.
e)	Primer design not optimal	Review primer design, see page 15.

References

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

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

Appendix: Adapter Primer Sequences and Multiple Cloning Sites

No tag Sense Primer

5'-ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAAC
ggTTTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA-'3

H_2N — Protein

Strep-tag Sense Primer

5'-ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAAC
ggTTTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA**ATg**TggTC
TCATCCgCAATTCgAAAAAAgCgCTgAAAACCTgATCgAAggCCgT'3

H_2N — MWSHPQFEKSAENLIEGR* — Protein

* Factor Xa cleavage site

6xHis tag Sense Primer

5'ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACgg
TTTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA**ATg**AAACATCA
TCACCATCACCACTCgACCCACgCgCATgTCgTAAAAAgCACCCAA-'3

H_2N—MKHHHHHHSTHAHAHVVKSTQ — Protein

No tag Antisense Primer

5'-ATgATATCACCggTgAATTCggATCCAAAAAACCCCTCAAgACCCgTTTAgA
ggCCCCAAggggTACAgATCTTggTTAgTTAgTTA**TTA**-3'

Protein — Stop — COOH

Strep-tag Antisense Primer

5'-ATgATATCACCggTgAATTCggATCCAAAAAACCCCTCAAgACCCgTTTA
gAggCCCCAAggggTACAgATCTTggTTAgTTAgTTA**TTA**TTTTTCgAATTgC
ggATgAgACCAggCAgA-3'

Protein — SAWSHPQFEK — Stop — COOH

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

6xHis tag Antisense Primer

5'-ATgATATCACCggTgAATTCggATCCAAAAAACCCCTCAAgACCCgTTTAgAggC
CCCAAggggTACAgATCTTggTTAgTTAgTTA**TTA**ATgATggTgATggTggTgACCCCA
-3'

Protein — WGHHHHHH — Stop — COOH

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

Sequencing primers

M13 forward (–20)	gTAAAACgACggCCAgT
M13 reverse (–21)	CAggAAACAgCTATgAC

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

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

EasyXpress Sense Primer Multiple Cloning Site

EcoRV *XhoI* *NotI* *NheI*

5'-ATGATATCTCGAGCGGCCGCTAGCT-3'
3'-TACTATAGAGCTCGCCGGCGATCGA-5'

EasyXpress Antisense Primer Multiple Cloning Site

BamHI *EcoRI* *SgrAI* *EcoRV*

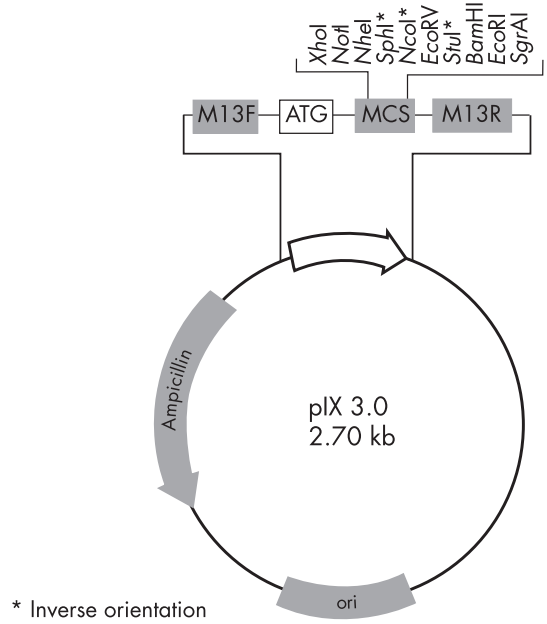
5'-TGGATCCGAATTCTCCGGTGATATCAT-3'
3'-ACCTAGGCTTAAGTGGCCACTATAGTA-5'

EasyXpress pIX3.0 Vector Multiple Cloning Site

XhoI *NotI* *NheI* *EcoRV* *BamHI* *EcoRI* *SgrAI*

5'-CTCGAGCGGCCGCTAGCATGCCATGGATATCAGGCCTGGATCCGAATTCACCGGTG-3'
3'-GAGCTCGCCGGCGATCGTACGGTACCTATAGTCCGGACCTAGGCTTAAGTGGCCAC-5'

Figure 6. The pIX3.0 Vector.



* As long as they do not have a restriction site within the PCR insert, these restriction enzymes can be added directly to the vector–insert ligation reaction to minimize the number of false positive clones (religations).

Ordering Information

Product	Contents	Cat. no.
EasyXpress Linear Template Kit Plus (20)	For 20 two-step PCRs: ProofStart DNA Polymerase, buffer, RNase-Free Water, Q-solution, XE-Solution, positive-control DNA, and optimized PCR primers	32723
EasyXpress pIX3.0 Vector	For protein expression in scaled-up in vitro translation reactions or *E. coli*: 25 µg vector DNA	32733
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
Related products		
EasyXpress Site-Specific Biotin Kit	For 5 x 25 µl reactions: *E. coli* extract, reaction buffer, RNase-Free Water, biotinyl-lysyl tRNA (amber), and positive-control DNA	32602
EasyXpress Protein Synthesis Mega Kit	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o methionine, methionine, RNase-Free Water, gel-filtration columns, and reaction flasks	32516
EasyXpress NMR Protein Synthesis Kit	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o Arg, Lys, Ser, Thr, Val (supplied as individual amino acids), RNase-Free Water, gel-filtration columns, and reaction flasks	32526

Ordering Information

Product	Contents	Cat. no.
EasyXpress NMR Protein Synthesis Kit –X*	For 2 x 5 ml reactions: *E. coli* extract, amino acid mix w/o X, reaction buffers, RNase-Free Water, gel-filtration columns, and reaction flasks	Varies
EasyXpress 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	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
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

* 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.
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
6xHis Protein Ladder	6xHis-tagged marker proteins (lyophilized, for 50–100 lanes on western blots)	34705
Plasmid DNA purification		
HiSpeed® Plasmid Midi Kit (25)	25 HiSpeed Midi Tips, 25 QIAfilter Midi Cartridges, 25 QIAprecipitator Midi Modules plus Syringes, Reagents, Buffers	12643
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

* Not available in all countries, please inquire.

Ordering Information

Product	Contents	Cat. no.
Reverse transcriptases		
Omniscript RT Kit (50)	For 50 reverse-transcription reactions: 200 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,* RNase-Free Water	205111
Sensiscript RT Kit (50)	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,* RNase-Free Water	205211

* Contains 5 mM each dNTP.

Notes

Notes

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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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