May 2008

QIAgenes *E. coli* Handbook

QIAgenes Expression Kit *E. coli*

For high-level expression of His-tagged proteins in *E. coli* systems

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Contents

Kit Contents

QIAgenes Expression Kit – *E. coli*	
Catalog No.	**Varies**
QIAgenes Expression Construct	10 µg
QIAgenes *E. coli* Positive Control (TNFα)	10 µg
Penta·His Antibody, BSA-free	3 µg
Ni-NTA Spin Columns	4

Storage Conditions

QIAgenes Expression Constructs and the **QIAgenes *E. coli* Positive Control** are supplied lyophilized in TE buffer and should be resuspended in a convenient volume (e.g., 50 µl) water and stored at –20°C.

Penta·His Antibodies should be stored lyophilized until they are to be used. They can be stored lyophilized for 1 year at 2–8°C. Dissolve Penta·His Antibodies (3 µg) in 15 µl water per tube (final concentration, 0.2 mg/ml). In solution they can be stored for 3 months at 2–8°C or for up to 6 months in aliquots at –20°C. Avoid repeated freezing and thawing.

Ni-NTA Spin Columns should be stored at 2–8°C. They can be stored under these conditions for up to 18 months without any reduction in performance.

Product Use Limitations

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

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 QIAgenes 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/goto/TechSupportCenter or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

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).

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, QIAgenes Kits are tested against predetermined specifications to ensure consistent product quality.

Safety Information

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

The following risk and safety phrases apply to the components of QIAgenes kits.

Ni-NTA Spin Columns

Contains nickel-nitrilotriacetic acid. Risk and safety phrases*: R22-40-42/43 S13-26-36-46

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

* R22: Harmful if swallowed; R40: Possible risks of irreversible effects; R42/43: May cause sensitization by inhalation and skin contact; S13: Keep away from food, drink, and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S46: If swallowed, seek medical advice immediately and show container or label.

Introduction

QIAgenes Expression Kits *E. coli* are used for high-level expression of recombinant proteins carrying an N-terminal 6xHis affinity tag in *E. coli* expression systems.

QIAgenes Expression Constructs *E. coli* (Figure 1) are plasmids containing expression-optimized, synthetic custom protein coding sequences* that are used for in vivo expression in *E. coli* cells or *E. coli*-based in vitro expression systems (e.g., EasyXpress *E. coli*-based kits). In addition, the kit contains a positive control plasmid for expressing a His-tagged control protein (TNFα), Ni-NTA Spin Columns, and Anti·His Antibodies for fast screening and analysis of target protein expression.

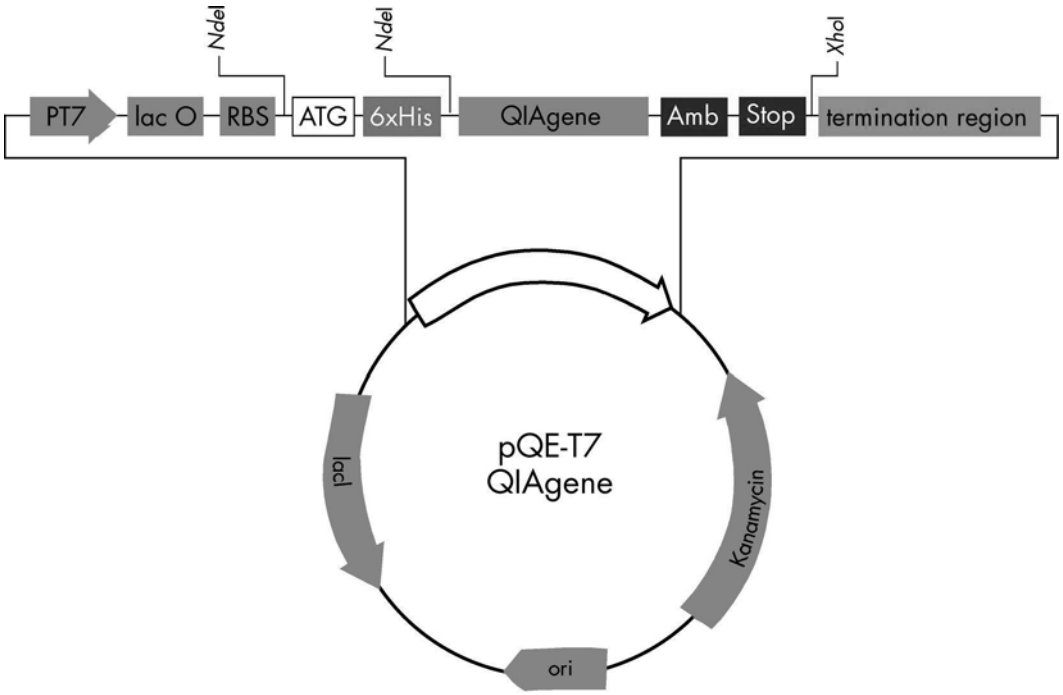

Figure 1. QIAgenes Expression Construct *E. coli* vector map. PT7: T7 promoter; **lac O**: lac operator; **RBS**: ribosome-binding site; **ATG**: start codon; **6xHis**: His tag; **QIAgene**: QIAgenes Expression Construct protein coding sequence; **Amb**: amber stop codon; **Stop**: stop codon; **ori**: origin of replication: **lacI**: lac repressor gene; **Kanamycin**: kanamycin resistance gene.

* Powered by GENEART® — the gene of your choice®

Optimized coding sequence for efficient expression

A limiting factor in efficient expression of eukaryotic proteins in prokaryotic systems is the difference in codon usage between bacterial and mammalian cells. In addition, secondary structures in mRNA can result in low translation efficiency and protein yields. Using a proprietary algorithm,* the human protein coding sequences in QIAgenes Expression Constructs are optimized with respect to *E. coli* codon usage, and are free of sequence repeats and other elements that build mRNA secondary structures, leading to more efficient expression (Figure 2).

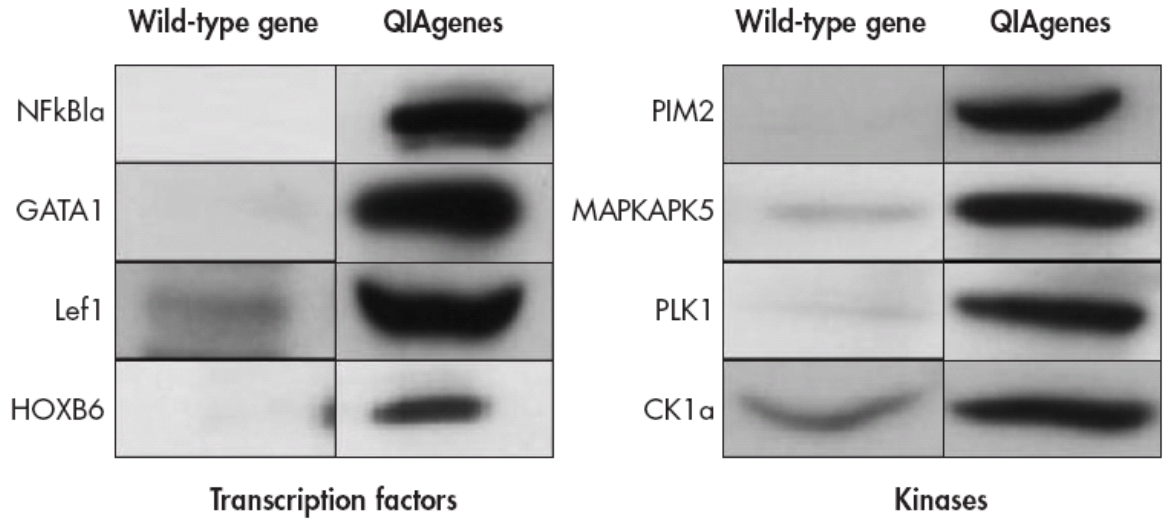

Figure 2. Optimized protein-coding sequences increase yields. The wild-type and an optimized QIAgenes coding sequence of the indicated proteins were cloned into QIAgene Expression Construct backbones, and expressed in parallel using an *E. coli*-based EasyXpress cell-free expression kit. Expression levels were visualized after separation of crude lysates by SDS-PAGE and western blotting using Penta·His Antibodies and chemiluminescent detection.

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QIAgenes Expression Constructs

QIAgenes Expression Constructs contain the following features:

- A T7 promoter system for efficient expression in B strain *E. coli* (e.g., BL21 [DE3])
- Optimized (for example, with respect to codon usage and mRNA stability and secondary structure) gene sequence, to ensure efficient expression
- A kanamycin resistance gene that enables selection of *E. coli* cells on media containing kanamycin
- An amber stop codon enabling cotranslational C-terminal biotin labeling using a biotinyl-lysyl tRNA (amber) (see page 15)

In addition, the protein's N-terminal tag sequence is optimized with respect to efficient exoproteolytic removal using the TAGZyme system (see page 28).

A map of the QIAgenes Expression Construct backbone can be found on the Product Sheet supplied with each QIAgenes Expression Kit and a detailed map and sequence can be found online at www.qiagen.com/protein/vectors.

Producing Recombinant Proteins in *E. coli* Cells Using QIAgenes Expression Constructs

QIAgenes expression constructs are based on the T7 promoter-driven pQE-T7 family of vectors. In pQE-T7 vectors, target genes are cloned into positions under the control of strong bacteriophage T7 transcription and translation signals, and expression is induced by providing a source of T7 RNA polymerase in the host cell. The source of the enzyme is a chromosomal copy of the RNA polymerase gene under lacUV5 control which is induced by the addition of IPTG or lactose. Strains carrying this gene are called λDE lysogens (= *E. coli* B strains), and include BL21(DE), C41(DE3), and Rosetta(DE3).

The production of T7 RNA polymerase is regulated by the presence of the *lac* repressor protein. During normal cell growth, this protein binds to operator sequences upstream of the T7 RNA polymerase gene and upstream of the QIAgene, preventing its transcription. The *lac* repressor protein is inactivated by adding isopropyl-β-D-thiogalactoside (IPTG) to the growth medium, allowing the host cell's RNA polymerase to transcribe the T7 RNA polymerase gene sequence downstream from the promoter. The T7 RNA polymerase can then attach to the T7 promoter upstream of the target gene on the QIAgene expression construct and initiate transcription of the target protein (Figure 3).

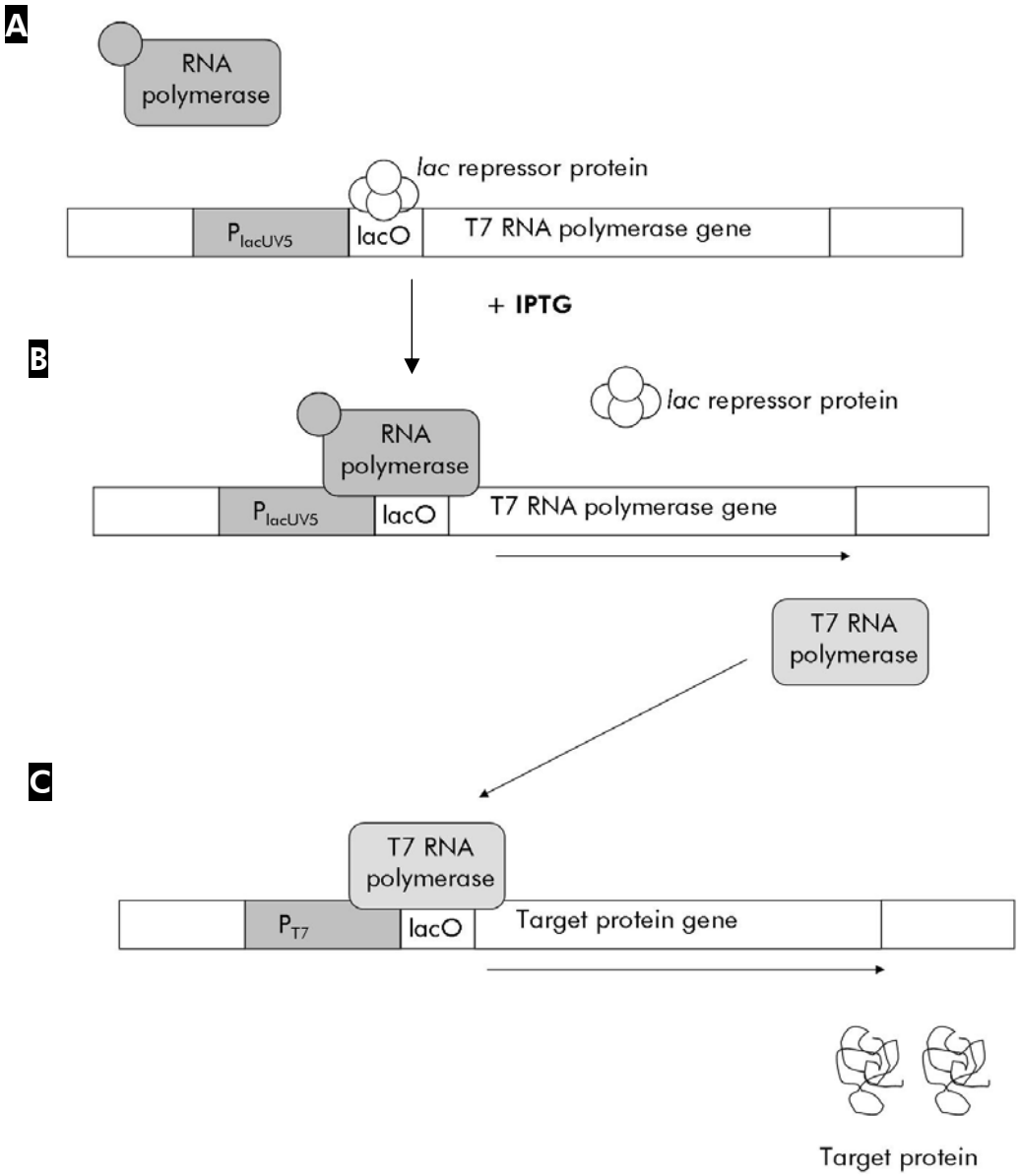


Figure 3. T7 promoter-driven expression. **A** *lac* repressor protein prevents expression of T7 RNA polymerase gene by cellular RNA polymerase. **B** Addition of IPTG dissociates *lac* repressor from operator allowing expression of T7 RNA polymerase gene. **C** T7 RNA polymerase binds to the T7 promoter on the QIAgenes Expression Construct and initiates expression of the target protein.

E. coli B strains carrying an additional pLysS or pLysE plasmid constitutively express lysozyme (a natural inhibitor of T7 RNA polymerase) and provide an additional level of regulation. Lysozyme inhibits T7 RNA polymerase potentially generated by "leaky" repression prior to induction. Tight regulation is of special importance if proteins toxic to *E. coli* are produced. Tighter regulation is provided by cells carrying the pLysE plasmid, where expression levels of lysozyme are higher than those carrying the pLysS plasmid. Adding the inducer IPTG (~1 mM) overcomes the inhibitory effect of the lysozyme protein present in the cell.

Introducing QIAgenes Expression Constructs into *E. coli* Cells

To produce recombinant protein, the QIAgenes Expression Construct must be introduced into competent *E. coli* B strain cells in a process called transformation. Protocols for producing competent *E. coli* cells and transforming them by heat-shocking the cells can be found on pages 52 and 30 respectively. Another commonly used method for transformation is electroporation. If using this method, consult your electroporator documentation for a suitable protocol.

Selecting and growing small-scale expression cultures

After transformation, an aliquot of the transformed cells is spread out onto an agar plate to allow isolation of individual *E. coli* colonies containing the QIAgenes Expression Construct. QIAgenes Expression Constructs carry a gene for kanamycin resistance, which allows *E. coli* containing these constructs to be selected for growth on agar plates or in cultures containing the antibiotic kanamycin.

Expression levels vary between different colonies of freshly transformed cells, and small-scale preparations permit the selection of clones delivering optimal expression rates. Small-scale expression and purification experiments are highly recommended and should be performed before proceeding with a large-scale preparation. In many cases aliquots of the cells can be lysed in a small volume of sample buffer and analyzed directly by SDS-PAGE or by western blot using an anti-His antibody (e.g., Penta·His) side-by-side with an uninduced control. Alternatively, expression can be analyzed by picking single colonies from the agar plate, growing them in liquid media, and purifying the expressed His-tagged protein. This provides a rapid way to judge the effects of varied growth conditions on expression levels and solubility of recombinant proteins.

Culture media

The media of choice for the growth of *E. coli* cells containing QIAgenes Expression Constructs are LB medium and its modifications, 2x YT, TB, or Super Broth, each containing 50 µg/ml kanamycin. Initially, it is advisable to try expression in all four media in parallel and to take a time course to monitor growth and expression after induction. Striking differences between the level of expression in different media and at different times are often seen.

Inducing protein expression

Efficient and controlled expression of target proteins from QIAgenes Expression Constructs in *E. coli* cells is regulated by the presence of the *lac* repressor protein (see page 10). During normal cell growth, this protein binds to the Lac operator (LacO) sequences upstream of the QIAgene and prevents recombinant protein expression. Expression is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and dissociates it from the operator, enabling T7 RNA polymerase to bind to the T7 promoter and subsequently to express the target protein.

This tight control of expression has the advantage that proteins that may have adverse effects on growing cells are not expressed, allowing cells to develop and grow normally until protein expression is induced.

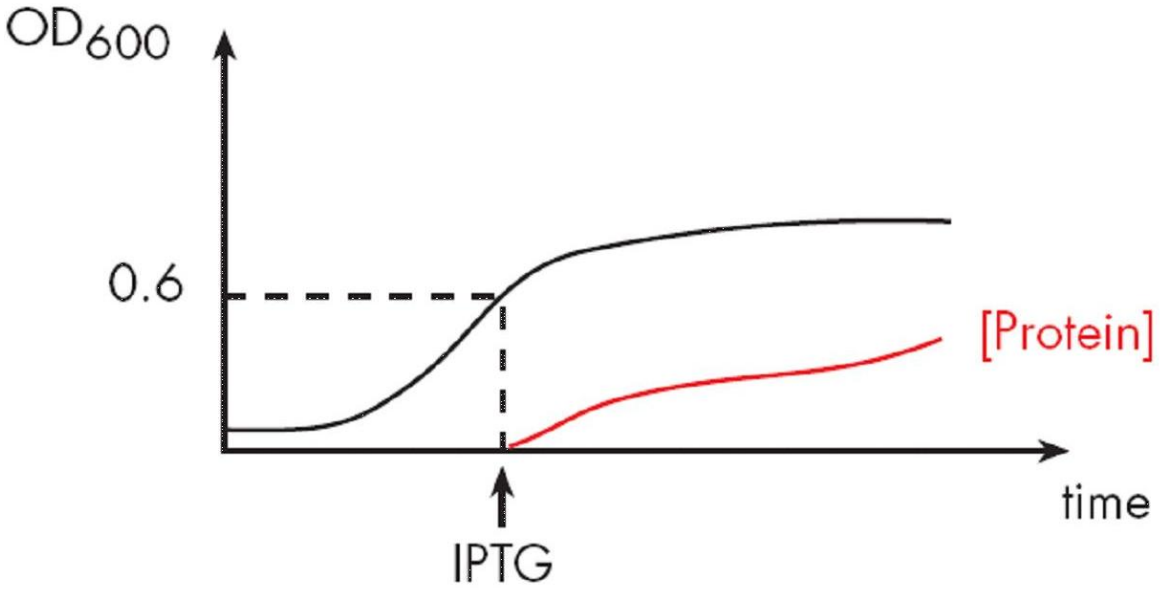


Figure 4. Induction of protein expression by addition of IPTG.

Time-course analysis of protein expression

To optimize the expression of a given protein, a time-course analysis of the level of protein expression is recommended (Figure 5). Intracellular protein content is often a balance between the amount of soluble protein in the cells, the formation of inclusion bodies, and protein degradation. By checking the amount of target protein present at various times after induction, the optimal induction period can be established.

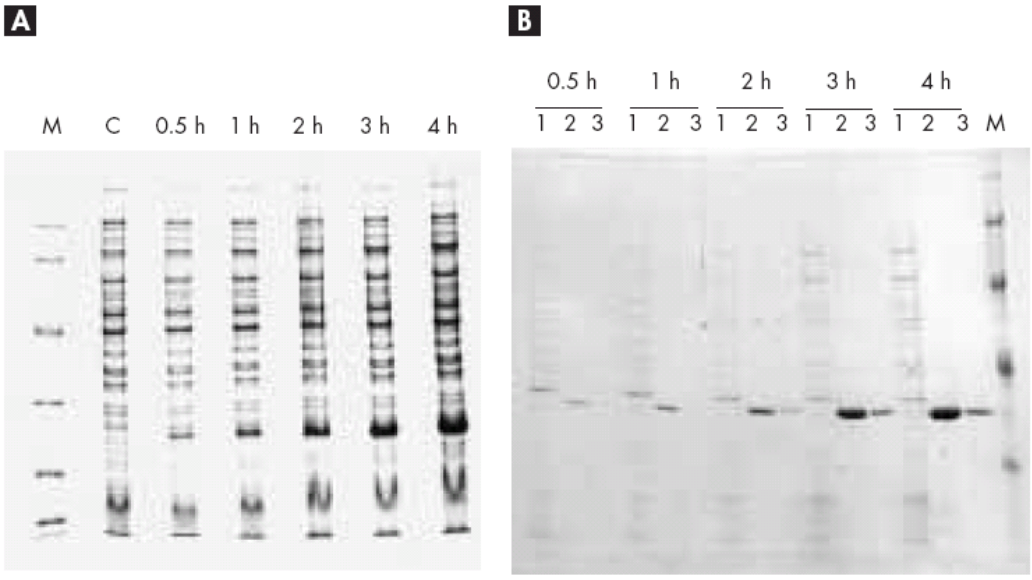


Figure 5. Time course of target protein expression. Expression of His-tagged DHFR was induced with 1 mM IPTG. Aliquots were removed at the times indicated and purified on Ni-NTA Agarose under denaturing conditions. Proteins were visualized by Coomassie staining. Yields per liter culture were 2.8, 5.5, 12.3, 33.8, and 53.9 mg, respectively.
A Crude cell lysate; **B** purification with Ni-NTA. **1**: flow-through, **2** & **3**: first and second eluates; **M**: markers; **C**: non-induced control.

Scaling up expression

Once an optimally expressing clone is identified, larger quantities of protein can be expressed using larger-scale cultures (reference 1). For a comprehensive guide to expressing and purifying His-tagged proteins see *The QIA*expressionist™, which is available for download at www.qiagen.com or on demand from your local Technical Services Department.

Subcloning QIAgenes Expression Constructs

Two cloning vectors — the QIAgenes N-terminal His tag Vector pQE-T7-1 (cat. no. 33013) and QIAgenes C-terminal His tag Vector pQE-T7-2 (cat. no. 33023) — are available for subcloning optimized protein-coding inserts or protein domains and expressing them in *E. coli*. Both vectors encode a 10xHis tag, whose extra length can lead to more efficient purification of membrane proteins in buffers containing detergent.

Strategies for cloning complete QIAgenes or isolated domains into pQE-T7-1 and 2 are described in detail in Appendix D (see page 55).

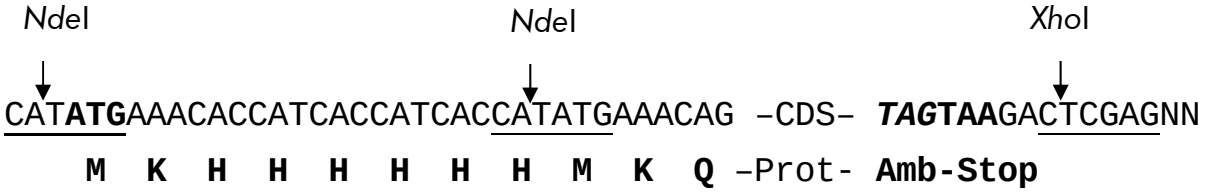


Figure 6. His-tag sequence in QIAgenes Expression Constructs *E. coli*. Start codon (ATG) in bold. Restriction sites underlined. **CDS**: QIAgene coding sequence. **Amb**: amber stop codon (italics).

Expression of an untagged protein can be performed after *Nde*I restriction, isolation of the QIAgene fragment from an agarose gel (separation from the *Nde*I/*Nde*I fragment), and vector religation.

Protein coding sequences in QIAgenes Expression Constructs *E. coli* have been designed to avoid certain restriction enzyme recognition sites that may consequently be used for cloning. The following enzymes do not cut in any QIAgene Expression Construct *E. coli*:

*Sac*II, *Not*I, *Sac*I, *Kpn*I, *Pst*I, *Xho*I, and *Nde*I

In Vitro (Cell-Free) Expression of Recombinant Proteins

Despite the optimization of QIAgenes Expression Constructs, in some cases very little or no expression of target protein may be seen in *E. coli* cells. This might be due to the toxicity of the expressed protein to the *E. coli* cells or degradation of the protein by intracellular proteases. In such cases, cell-free expression is an option.

QIAgenes *E. coli* Expression Constructs are compatible with QIAGEN EasyXpress *E. coli*-based kits. Using the EasyXpress system enables production of up to 1 mg protein per ml reaction volume in just one hour (2). Reactions are easily scalable (up to mg amounts for structural studies) and no special equipment is required. Labeling of proteins (e.g., with selenomethionine or isotope-labeled amino acids for structural analysis by x-ray crystallography or NMR) is also facilitated by the compact reaction volumes. For more information on the EasyXpress System, visit www.qiagen.com or contact your local Technical Services Department.

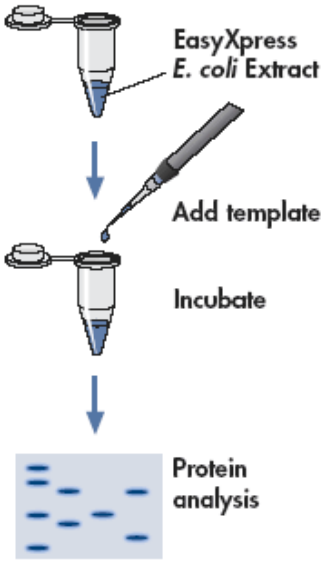


Figure 7. In vitro translation of recombinant proteins.

Cotranslational biotin labeling of recombinant proteins

Using the unique EasyXpress Site-Specific Biotin Kit (patent pending, cat. no. 32602) in conjunction with a QIAgenes Expression Construct enables insertion of a biotin label at the C-terminus of the expressed protein. This insertion is accomplished using a synthetic aminoacylated tRNA carrying a biotinylated lysine residue and an anticodon that recognizes the UAG amber stop codon (Figure 8). Site-specific biotinylation facilitates immobilization of the proteins on Streptavidin supports for interaction studies (3).

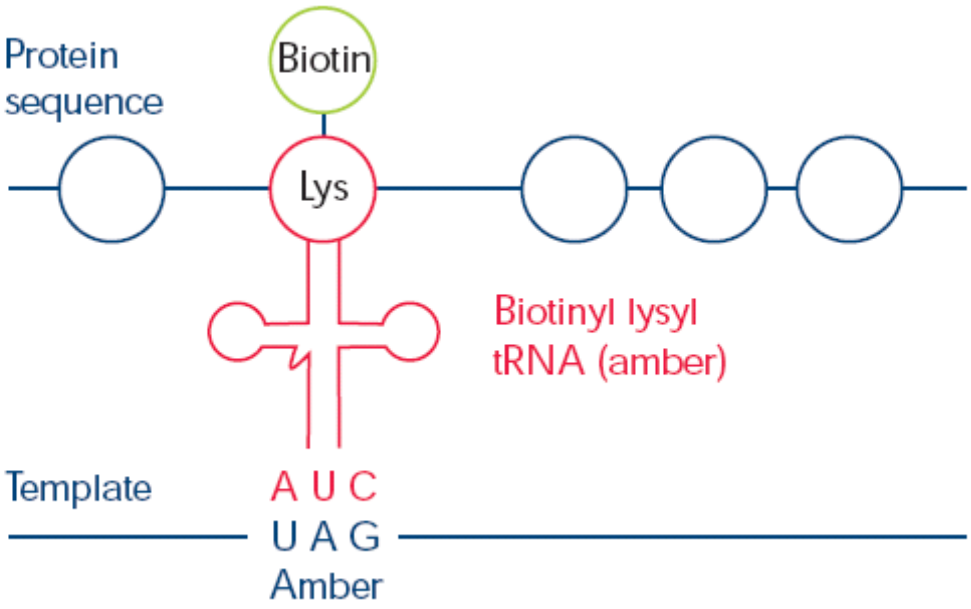


Figure 8. Schematic representation of biotin incorporation using the EasyXpress Site-Specific Biotin Kit.

The EasyXpress *E.coli* lysates in the EasyXpress Site-Specific Biotin Kit are treated to reduce the levels of release factor 1 (RF1), a protein whose presence would cause translation termination at the amber codon. This enables translation to continue through the amber codon and incorporation of the biotinylated residue at the C-terminus. Incorporation of biotin occurs at a stoichiometry of 1:1.

The *E. coli* lysate used for in vitro translation with the EasyXpress Site-Specific Biotin Kit has been adapted to the specific requirements of site-specific labeling. The lysate possesses very fast synthesis kinetics and the levels of some translation factors important for efficient synthesis of site-specifically labeled proteins have been modified. Endogenous biotinylated proteins normally present in prokaryotic lysates have been removed, greatly facilitating assays that make use of the biotin moiety for protein detection.

Using the EasyXpress Site-Specific Biotin Kit, up to 5 µmol (typically 150 µg/ml) biotin-labeled protein can be synthesized within 30 minutes. The synthesis can be easily scaled up or down. The amount of protein synthesized increases linearly with increased reaction volume. For more information on cotranslational biotin labeling, refer to the *EasyXpress Site-Specific Biotin Labeling Handbook*, available for download online at www.qiagen.com or from your local Technical Services Department.

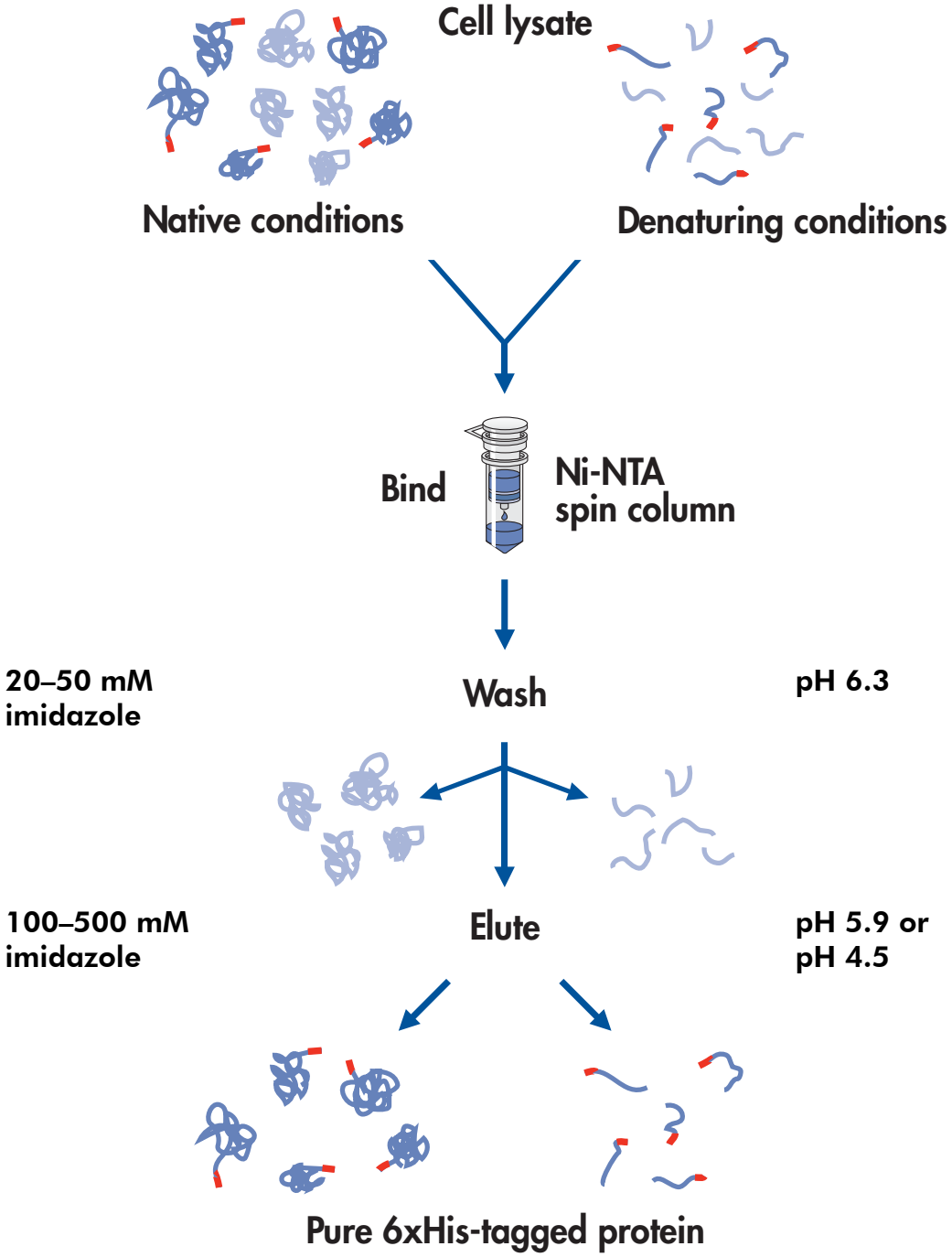
Purifying His-tagged Proteins

Ni-NTA Spin Columns supplied with the QIAgenes Expression Kit provide a simple method for rapid screening and purification of His-tagged proteins from small-scale expression cultures. They are based on the remarkable selectivity of Ni-NTA (4) for recombinant proteins carrying a small affinity tag consisting of consecutive histidine residues, the His tag (5). Ni-NTA Spin Columns are based on Ni-NTA Silica, a unique and versatile metal chelate chromatography material, packaged in ready-to-use spin columns. They allow rapid purification of proteins from crude cell lysates under either native or denaturing conditions (see flowchart on page 18). The one-step procedure allows purification of up to 300 µg His-tagged protein per column in as little as 30 minutes.

Figure 9. The QIAcube

Fully automated purification of His-tagged proteins on the QIAcube

Purification of His-tagged proteins can be fully automated on the QIAcube (Figure 9). The innovative QIAcube uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into laboratory workflows. Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute) and delivers high-purity His-tagged proteins (Figure 10).


Cell lysate
Native conditions
Denaturing conditions
Bind
Ni-NTA
spin column
20–50 mM
imidazole
Wash
pH 6.3
100–500 mM
imidazole
Elute
pH 5.9 or
pH 4.5
Pure 6xHis-tagged protein

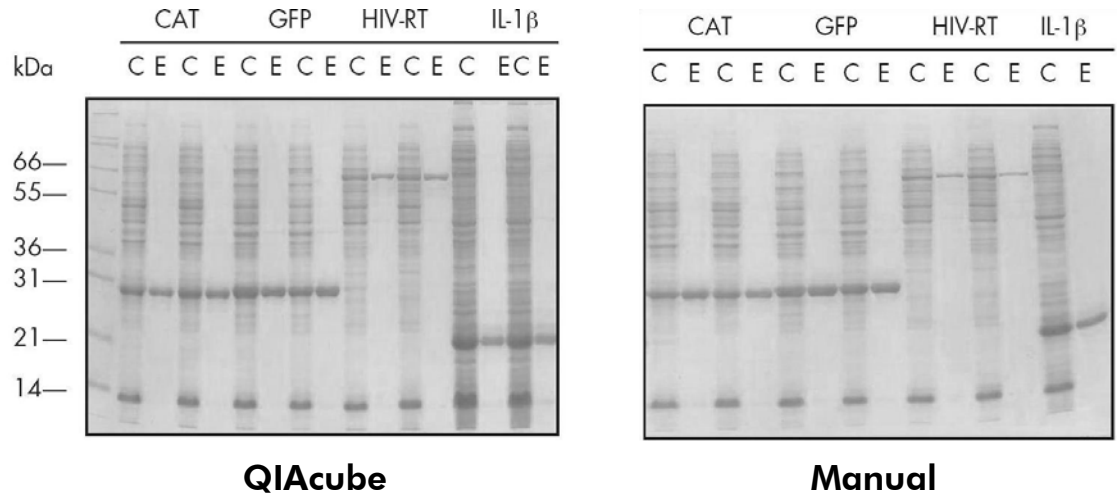


Figure 10. Efficient automated and manual purification of His-tagged proteins using Ni-NTA spin columns. The indicated proteins were purified in duplicate under native conditions using Ni-NTA Spin Columns from cleared *E. coli* cell lysates derived from 5 ml LB cultures either manually or in an automated procedure on the QIAcube. **CAT**: chloramphenicol acetyl transferase; **GFP**: Green fluorescent protein; **HIV-RT**: Human immunodeficiency virus reverse transcriptase; **IL-1b**: Interleukin-1 beta. **M**: markers; **C**: cleared lysate (2 µl loaded per lane); **E**: elution fraction (3 µl loaded per lane).

Purification Under Native or Denaturing Conditions

The decision whether to purify His-tagged proteins under native or denaturing conditions depends on protein location and solubility, the accessibility of the His tag, the downstream application, and whether biological activity must be retained. Furthermore, if efficient renaturation procedures are available, denaturing purification and subsequent refolding may be considered. To assess protein solubility and the optimal purification procedure, we recommend using the four Ni-NTA Spin Columns in QIAgenes Expression Kits to perform both native and denaturing purification of the target and control proteins. Protocols for purification of His-tagged proteins from *E. coli* lysates under both denaturing and native conditions are available for the QIAcube. Visit www.qiagen.com/MyQIAcube for more details.

Purification under native conditions

If purification under native conditions is preferred or necessary, the His-tagged protein must be soluble. However, even when most of the protein is present in inclusion bodies, there is generally some soluble material that can be purified in its native form. The potential for unrelated, nontagged proteins to interact with the Ni-NTA resin is usually higher under native than under denaturing conditions. This is reflected in the larger number of proteins that appear in the first wash. Nonspecific binding is reduced by including a low concentration of imidazole in the lysis and wash buffers.

In rare cases the His tag is hidden by the tertiary structure of the native protein, so that soluble proteins require denaturation before they can be purified on Ni-NTA. As a control, a parallel purification under denaturing conditions should always be carried out. Prepare a lysate under native conditions and centrifuge; collect supernatant (soluble protein) and purify under native conditions; dissolve the pellet from the centrifugation step in a chaotropic buffer (e.g., Buffer B/7M urea), centrifuge and collect supernatant (insoluble protein) and purify under denaturing conditions. If purification is only possible under denaturing conditions, the tag can generally be made accessible by moving it to the opposite terminus of the protein by subcloning the expression cassette into the QIAgenes C-terminal His tag Vector pQE-T7-2 (cat. no. 33023).

For purification of membrane proteins detergents may be required for resolubilization from membranes. Protocols for purification of membrane proteins are available in the literature or from QIAGEN upon request. For more information, please see our Technical Support center at www.qiagen.com/goto/TechSupportCenter or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Purification under denaturing conditions

High levels of expression of recombinant proteins in a variety of expression systems can lead to the formation of insoluble aggregates; in *E. coli*, these are known as inclusion bodies. The denaturing buffers listed in Appendix A completely solubilize inclusion bodies and His-tagged proteins. Under denaturing conditions, the His tag on a protein will be fully exposed so that binding to the Ni-NTA matrix will improve and the efficiency of the purification procedure will be maximized by reducing the potential for nonspecific binding.

His-tagged proteins purified under denaturing conditions can be used directly, or may have to be renatured and refolded. Protein renaturation and refolding can be carried out on the Ni-NTA column itself prior to elution, or in solution; suggestions can be found in *The QIA*expressionist, which is available for download at www.qiagen.com or on demand from your local Technical Services Department.

Scaling up purification

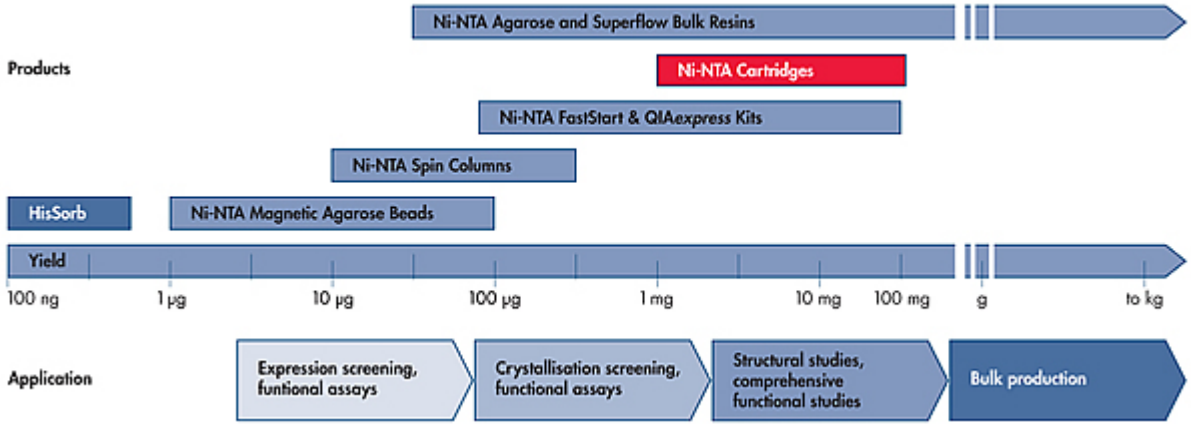


Figure 11. Ni-NTA matrices for His-tagged protein purification.

QIAGEN offers a range of Ni-NTA matrices for His-tagged protein purification on any scale (see Figure 11). Yields and purities are consistent across the complete range of products, enabling problem-free scale up from expression screening to preparative purifications.

Table 1. Compatibility of reagents with Ni-NTA

Reagent	Effect	Comments
Buffer reagents		
Tris, HEPES, MOPS	Buffers with secondary or tertiary amines may reduce nickel ions.	Up to 100 mM can be used, however sodium phosphate or phosphate-citrate buffer is recommended.
Chelating reagents		
EDTA, EGTA	Strips nickel ions from resin.	Up to 1 mM has been used successfully in some cases, but care must be taken.
Sulfhydril reagents		
β-mercaptoethanol	Prevents disulfide cross-linkages. Can reduce nickel ions at higher concentration.	Up to 20 mM can be used. Do not store resin under reducing conditions.
DTT, DTE	At high concentrations (>1 mM) resin may turn reversibly brown due to nickel reduction. Up to 10 mM has been tested and shown not to compromise purification or increase nickel leaching.	Up to 10 mM DTT has been used successfully. Do not store resin under reducing conditions.
TCEP	Prevents disulfide cross-linkages.	Up to 1 mM tested successfully. Do not store resin under reducing conditions.

Reagent	Effect	Comments
Nonionic detergents		
n-Hexadecyl-β-D-maltoside	Removes background proteins and nucleic acids, resolubilizes membrane proteins from membrane compartments.	0.0003%*
n-Tetradecyl-β-D-maltopyranoside		0.005%*
n-Tridecyl-β-D-maltopyranoside		0.016%*
Brij 35		0.1%*
Digitonin		0.6%*
Cymal 6, n-Nonyl-β-D-glucopyranoside, n-Decyl-β-D-maltopyranoside, n-Dodecyl-β-D-maltoside, C12-E9		1%*
n-Octyl-β-D-glucopyranoside		1.5%*
Triton®, Tween®, NP-40		Up to 2% can be used.
Zwitterionic detergents		
Fos-Choline 16		0.05%*
Dodecyldimethylphosphine oxide		0.15%*
Cationic detergents		Up to 1% can be used.
CHAPS		Up to 1% can be used.

* Highest concentration tested at QIAGEN. Maximum concentration compatible with Ni-NTA may be higher.

Reagent	Effect	Comments
Anionic detergents (SDS, sarkosyl)		Not recommended, but up to 0.3% has been used successfully in some cases.
Triton X-114	Removes endotoxins	Up to 2% can be used.
Denaturants		
GuHCl	Solubilizes proteins	Up to 6 M.
Urea		Up to 8 M.
Amino acids		
Glycine		Not recommended.
Glutamine		Not recommended.
Arginine		Not recommended.
Histidine	Binds to Ni-NTA and competes with histidine residues in the His tag. Elution with histidine can help to reduce aggregation of eluted protein.	Can be used at low concentrations (1–2 mM) to inhibit non specific binding and, at higher concentrations (>20 mM), to elute the His-tagged protein from the Ni-NTA matrix.
Other additives		
NaCl	Prevents ionic interactions.	Up to 2 M can be used, at least 300 mM should be used.
$MgCl_2$		Up to 4 M.
$CaCl_2$		Up to 5 mM.
Glycerol	Prevents hydrophobic interaction between proteins, stabilizes proteins.	Up to 50%.

Reagent	Effect	Comments
Ethanol	Prevents hydrophobic interactions between proteins.	Up to 20%.
BugBuster® Protein Extraction Reagent		Use as recommended.
Imidazole	Binds to Ni-NTA and competes with histidine residues in the His tag.	Can be used at low concentrations (20 mM) to inhibit non specific binding and, at higher concentrations (>100 mM), to elute the His-tagged protein from the Ni-NTA matrix.
Sodium bicarbonate		Not recommended.
Hemoglobin		Not recommended.
Ammonium		Not recommended.
Citrate	Buffer	Up to 60 mM has been used successfully.

Preparation of the cell lysate and protein binding under native conditions

Before purifying proteins under native conditions, it is important to determine how much of the protein is soluble in the cytoplasm and how much is in insoluble precipitates or inclusion bodies. Parallel purification under denaturing conditions is recommended.

Because of variations in protein structure that can interfere with binding, it is difficult to provide an exact protocol for purification of tagged proteins under native conditions. However, some general guidelines are helpful to optimize the purification procedure:

- Since there is often a higher background under native conditions, low concentrations of imidazole in the lysis and washing buffers are recommended. Binding of nontagged contaminating proteins is inhibited, leading to greater purity in fewer steps.

- For most proteins, up to 10–20 mM imidazole can be used without affecting the yield. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM.
- Addition of β-mercaptoethanol (up to 20 mM) or DTT (up to 10 mM) reduces any disulfide bonds which may have formed between contaminating proteins and the His-tagged protein. Under some circumstances, however, especially when the proteins have a strongly reducing character, the resin may turn brown due to nickel reduction. This does not usually compromise purity or quality of the purified protein.
- Cell pellets frozen for at least 30 minutes at –20°C can be lysed by resuspending in lysis buffer and addition of lysozyme (1 mg/ml) and Benzonase® Nuclease (3 Units /ml culture volume). Fresh, i.e., unfrozen pellets require sonication or homogenization in addition to the lysozyme/Benzonase treatment. Detergent-based lysis buffer formulations may also be used, but are usually somewhat less efficient.
- All buffers should have sufficient ionic strength to prevent nonspecific interactions between proteins and the Ni-NTA matrix. The minimum salt concentration during binding and washing steps should be 300 mM NaCl. The maximal concentration is 2 M NaCl.
- For control purposes, QIAgenes control protein can be expressed in any *E. coli* B strain (e.g., BL21 [DE3]). The control plasmid encodes His-tagged TNFα (tumor necrosis factor alpha) which has an apparent molecular weight on SDS-PAGE gels of 21 kDa.

Preparation of the cell lysate and protein binding under denaturing conditions

Cells can be lysed in either 6 M GuHCl or 7–8 M urea. It is preferable to lyse the cells in the milder denaturant, urea, so that the cell lysate can be analyzed directly on an SDS polyacrylamide gel. GuHCl is a more efficient solubilization and cell lysis reagent, however, and may be required to solubilize some proteins.

Estimating expression level

It is important to estimate the expression level of your protein, for example using SDS-PAGE (Figure 3). For proteins that are expressed at very high levels (>10 mg per liter assuming 10^9 bacterial cells per ml, i.e., equivalent to an expression level of >12% of total cellular protein), the cell lysate may be concentrated 10-fold relative to the original culture volume. The pellet of a 10-ml culture, for example, should be lysed in 1 ml lysis buffer. For an expression level of 10 mg per liter, 600 µl of the 10x cell lysate in Buffer B would contain approximately 60 µg of His-tagged protein.

For lower expression levels (2–5 mg/liter) 25x cell lysates (600 µl cell lysate = 30–75 µg) should be prepared for loading onto the Ni-NTA spin column. If expression levels are expected to be lower than 1 mg per liter, the cell lysate should be prepared at a 50-fold concentration.

Microspin procedure summary

The purification procedure can be divided into three stages: preparation of the cell lysate and binding of the His-tagged protein to Ni-NTA silica, washing, and elution of the His-tagged protein. Up to 600 µl of cell lysate is loaded onto a Ni-NTA spin column and centrifuged for 5 minutes to bind His-tagged proteins to the Ni-NTA silica. Most of the nontagged proteins flow through. Residual contaminants and nontagged proteins are removed by washing with buffers of slightly reduced pH or with buffers containing a low concentration of imidazole. Purified protein is eluted in a volume of 100–300 µl.

Protein elution

Elution of the tagged proteins from the column can be achieved either by reducing the pH, or by competition with imidazole. Monomers are generally eluted at approximately pH 5.9 or with imidazole concentrations greater than 100 mM, whereas multimers elute at around pH 4.5 or 200 mM imidazole. Elution using Buffer E (pH 4.5) or buffers containing ≥250 mM imidazole (pH 8) is therefore recommended. 100 mM EDTA elutes all bound protein and strips metal from the resin.

Using a Ni-NTA spin column up to ~300 µg of high-purity (~90%) His-tagged protein can be prepared. Actual yields and purity will vary depending on the size and expression level of the recombinant protein, as well as the viscosity of the lysate. The recommended elution volume is 200–300 µl. To obtain even higher protein concentrations, elution volumes can be reduced to 100–200 µl.

Removing the His tag from Proteins Expressed Using QIAgenes Expression Constructs

His-tag amino acid sequences coded by QIAgenes Expression Constructs can be completely and efficiently removed using the TAGZyme system, generating the native protein free of any vector-encoded amino acid (Figure 11). Typical protein applications where tag removal may be advantageous include structural studies by crystallography (6) and NMR (7) or the production of therapeutic proteins (1). The exoproteolytic mode of action of the TAGZyme system ensures that no intramolecular cleavage takes place, guaranteeing homogeneity. After incubation with TAGZyme enzymes, pure detagged target proteins are recovered in the flow-through fraction of a subtractive Ni-NTA purification. Unprocessed target proteins, tag fragments, and the His-tagged TAGZyme enzymes are retained on the column. For more information on the TAGZyme affinity tag removal system, visit www.qiagen.com/tagzyme .

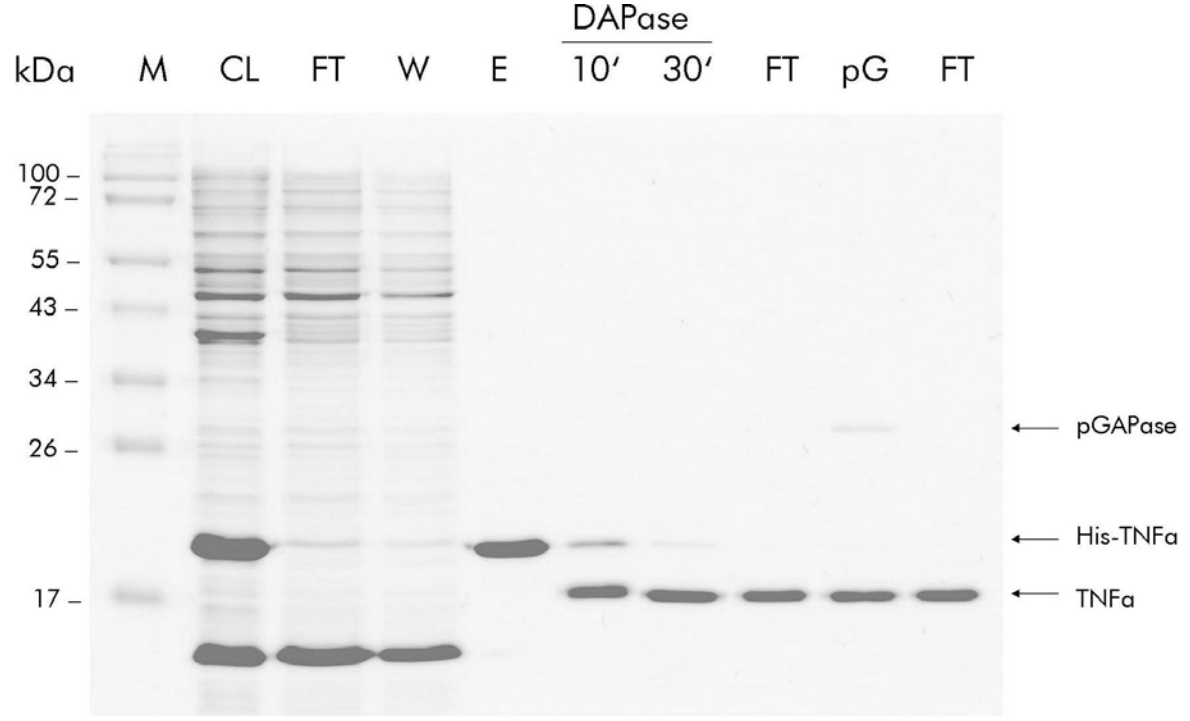


Figure 12. Efficient His-tag removal. The QIAgenes *E. coli* Positive Control expressing His-tagged TNFα was expressed in *E. coli* and purified using Ni-NTA Superflow. The His tag was removed by initial incubation with DAPase™ and excess Qcyclase™ for 30 min after which His-tagged DAPase and Qcyclase were removed by subtractive IMAC. TNFα with a pyroglutamate residue at the N-terminus was collected in the flow-through. The N-terminal pyroglutamate was removed by incubation with His-tagged pGAPase which itself was separated from native sequence TNFα by another round of subtractive IMAC. **M**: markers; **CL**: cell lysate; **FT**: flow-through; **W**: wash; **E**: eluate; **pG**: pGAPase reaction.

Analyzing Recombinant Protein Expression and Purification

Expression analysis is most easily performed using SDS polyacrylamide gel electrophoresis (SDS-PAGE). Using this technique, proteins are separated according to their size in a polyacrylamide gel matrix. After separation, proteins can be visualized using a universal staining technique, such as silver or Coomassie® staining.

If proteins are poorly resolved or present in low amounts, individual proteins can be visualized using antibodies, in a technique called western blotting. This technique involves transfer of proteins from the gel to a nitrocellulose or PVDF membrane and probing with antibodies specific to a protein or affinity-tag epitope. QIAgenes Expression Kits contain the Penta·His Antibody which recognizes an epitope of five consecutive histidine residues. This antibody can be used for immunodetection of His-tagged protein expressed from any QIAgenes Expression Construct.

6xHis Protein Ladder

When analyzing the expression of His-tagged proteins, the 6xHis Protein Ladder (cat. no. 34705) serves as a molecular weight standard and a positive control for western blotting. The His Protein Ladder consists of five His-tagged proteins ranging from 15 to 100 kDa in size.

Protocol: Transformation of Competent *E. coli* Cells

Materials and equipment to be supplied by user*

- Competent *E. coli* BL21 strain cells, e.g., BL21 (DE3) Competent Cells (Sigma, cat. no. B 8808), BL21 (DE3) pLysS Competent Cells (Sigma, cat. no. B 8933), BL21 (DE3) pLysE Competent Cells (Sigma, cat. no. B 9058). Alternatively, competent cells can be produced using the protocol in Appendix B, page 52.
- QIAgenes Expression Construct DNA in solution (refer to Product Sheet for resolubilization protocol)
- LB agar plates containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid)
- Psi broth
- Heating block or water bath set to 42°C

For composition of media and solutions, see Appendix A, page 48.

Procedure

1. **Transfer an aliquot of the QIAgenes Expression Construct DNA (1 µl or less) into a cold sterile 1.5 ml microcentrifuge tube, and keep it on ice.**
1. **Thaw an aliquot of frozen competent *E. coli* cells on ice.**
2. **Gently resuspend the cells and transfer 100 µl of the cell suspension into the microcentrifuge tube with the QIAgenes Expression Construct DNA, mix carefully, and keep it on ice for 20 min.**
3. **Transfer the tube to a 42°C water bath or heating block for 90 sec.**
4. **Add 500 µl Psi broth to the cells and incubate for 60–90 min at 37°C.** Shaking increases transformation efficiency.
5. **Plate out 50, 100, and 200 µl aliquots on LB-agar plates containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid). Incubate the plates at 37°C overnight.**

* 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.

Positive control to check transformation efficiency:

6. **Transform competent cells with 1 ng of the QIAgenes control plasmid (undigested) in 20 µl of TE. This plasmid expresses the 20 kDa protein 6xHis-TNFα. Plate 1/100 and 1/10 dilutions of the transformation mix (diluted in prewarmed Psi broth) as well as undiluted transformation mix on LB-agar plates containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid). The cells should yield 10^6 transformants per microgram of plasmid.**

Negative control to check antibiotic activity:

7. **Transform cells with 20 µl of TE. Plate at least 200 µl of the transformation mix on a single LB agar plate containing the appropriate antibiotics.**

Protocol: Growth of Expression Cultures

Materials and equipment to be supplied by user*

- LB medium: 10 g/liter bacto-tryptone, 5 g/liter bacto yeast extract, and 5 g/liter NaCl containing containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid)
- IPTG stock solution: 1 M IPTG (e.g., QIAGEN cat. no. 129921) in water, sterilize by filtration, store at –20°C

Protocol

1. **Inoculate 10 ml of LB medium containing the appropriate antibiotics with a fresh bacterial colony harboring the QIAgenes Expression Construct. Grow at 37°C overnight.**
1. **Dilute the non-induced overnight culture 1:60 (e.g., inoculate 30 ml medium with 500 µl overnight culture) with fresh LB medium containing the appropriate antibiotics. Grow at 37°C with vigorous shaking until the OD_{600} reaches 0.6–1.0.**

   For control purposes, QIAgenes control protein can be expressed in any *E. coli* B strain (e.g., BL21 [DE3]). The control plasmid encodes His-tagged TNFα (tumor necrosis factor alpha) which has an apparent molecular weight on SDS-PAGE gels of 21 kDa.

   The required volume of expression culture is mainly determined by the expression level, solubility of the protein, and purification conditions. For purification of proteins expressed at low levels, especially under native conditions, the minimum cell culture volume should be approximately 50 ml.
2. **Add IPTG to a final concentration of 1 mM and grow the culture at 37°C with vigorous shaking for 4 hours.**

   For proteins which are very sensitive to protein degradation, the induction time should be reduced and a time course of expression should be determined. In some cases, addition of 0.1–1 mM PMSF after induction is recommended to inhibit PMSF sensitive proteases. PMSF treatment can result, however, in a lower expression level.

* 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.

3. **Harvest the cells by centrifugation at 4000 x *g* for 15 min.**
4. **Store cell pellet at –20°C if desired or process immediately as described for purification under native conditions (page 34) or denaturing conditions (page 37).**

Protocol: Protein Purification under Native Conditions from *E. coli* Cell Lysates Using Ni-NTA Spin Columns

Materials and equipment to be supplied by user*

- Lysis Buffer (NPI-10): 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0
- Wash Buffer (NPI-20): 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0
- Elution Buffer (NPI-500): 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0
- Benzonase® endonuclease 25 U/µl (e.g., Novagen cat. no. 70664-3)
- Lysozyme (e.g., Roche cat. no. 837059) stock solution 10 mg/ml in water. Sterilize by filtration and store in aliquots at –20°C.

This protocol is suitable for use with frozen cell pellets. Cell pellets frozen for at least 30 minutes at –20°C can be lysed by resuspending in lysis buffer and adding Benzonase® Nuclease (3 Units /ml culture volume). Fresh (i.e., not frozen) pellets require sonication or homogenization in addition to the addition of 3 Units/ml culture volume Benzonase® Nuclease and 1 mg/ml culture volume lysozyme.

Important notes

- To ensure efficient binding, it is important not to exceed 270 x *g* (approx. 1600 rpm) when centrifuging Ni-NTA spin columns. At higher forces, even if the binding kinetics are high, the time the lysate is in contact with the resin is not sufficient for effective binding.
- Since silica is not inert in solutions of high pH, buffers with pH >8.4 should not be used with the Ni-NTA silica material.
- Avoid high concentrations of buffer components containing strong electron-donating groups (e.g., glycine, arginine, Tris; see Table 1).
- Cells should be lysed without the use of strong chelating agents such as EDTA or ionic detergents (e.g., SDS). Although low levels of these reagents have been used successfully, leaching may occur, and performance may be diminished.

* 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.

- Please take into account that the time needed for the centrifugation step during protein binding is influenced by the viscosity of the cleared lysate. For very concentrated cell lysates, it may be necessary to extend the centrifugation time to 5 or 10 min at 270 x *g* (approx. 1600 rpm).
- The spin columns should be centrifuged with an open lid to ensure that the centrifugation step is completed after 2 min. Under native conditions, it may be preferable to centrifuge with a closed lid to reduce the flow rate thereby extending binding time.
- Some proteins may be subject to degradation during cell harvest, lysis, or even during growth after induction. In these cases, addition of PMSF (0.1–1 mM) or other protease inhibitors is recommended. PMSF treatment during cell growth may result, however, in lower expression levels. Under native purification conditions it is best to work quickly and at 4°C at all times.

Protocol

1. **Resuspend a pellet derived from 5 ml cell culture volume in 630 µl Lysis Buffer (NPI-10). Add 70 µl Lysozyme Stock Solution (10 mg/ml) and add 3 Units/ml culture volume Benzonase® Nuclease (i.e., for cell pellets from 5 ml cultures, add 15 Units Benzonase® Nuclease).**

   Cells from 5 ml culture are usually used, but culture volume used depends on protein expression level. Resuspending pellet in 700 µl buffer will allow recovery of a volume of cleared lysate of approx. 600 µl.

   Do not use pellets from culture volumes greater than 70 ml. If larger culture volumes shall be processed resuspend in 2 x 630 µl and load the supernatant in 2 portions of 600 µl successively. By adding 10 mM imidazole, binding of nontagged contaminating proteins is inhibited, leading to greater purity in fewer steps. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM.

2. **Incubate on ice for 15–30 min.**
3. **Centrifuge lysate at 12,000 x *g* for 15–30 min at 4°C. Collect supernatant.**

   Save 20 µl of the cleared lysate for SDS-PAGE analysis.

4. **Equilibrate the Ni-NTA spin column with 600 µl Buffer NPI-10. Centrifuge for 2 min at 890 x *g* (approx. 2900 rpm).**

   The spin columns should be centrifuged with an open lid to ensure that the centrifugation step is completed after 2 min.

   By adding 10 mM imidazole, the binding of nontagged contaminating proteins is minimized, leading to greater purity in fewer steps. If the tagged protein does not bind under these conditions the amount of imidazole should be reduced to 1–5 mM.

5. **Load up to 600 µl of the cleared lysate containing the His-tagged protein onto the pre-equilibrated Ni-NTA spin column. Centrifuge for 5 min at 270 x *g* (approx. 1600 rpm), and collect the flow-through.**

   To ensure efficient binding it is important not to exceed 270 x g (approx. 1600 rpm) when centrifuging Ni-NTA spin columns. At higher forces, even if the binding kinetics are high, the time the lysate is in contact with the resin is not sufficient for effective binding.

   The spin columns can be centrifuged with an open lid to ensure that the centrifugation step is completed after 5 min, but under native conditions, it may be preferable to centrifuge with a closed lid to reduce the flow rate thereby extending binding time.

   Please take into account that the time needed for the centrifugation step during protein binding is influenced by the viscosity of the cleared lysate. For very concentrated cell lysates, it may be necessary to extend the centrifugation time to 5 or 10 min at 270 x g (approx. 1600 rpm).

   Save the flow-through for analysis by SDS-PAGE to check binding efficiency.

6. **Wash the Ni-NTA spin column twice with 600 µl Buffer NPI-20. Centrifuge for 2 min at 890 x *g* (approx. 2900 rpm).**

   The number of wash steps required to obtain highly pure protein is determined primarily by the expression level of the His-tagged protein. When the expression level is high, 2 washes are usually sufficient for removal of contaminants. For very low expression levels or highly concentrated lysates, 3 wash steps may be required to achieve high purity.

   Save the flow-through (wash fractions) for analysis by SDS-PAGE to check the stringency of the wash conditions.

7. **Elute the protein with 300 µl Buffer NPI-500. Centrifuge for 2 min at 890 x *g* (approx. 2900 rpm), and collect the eluate.**

   Most of the His-tagged protein (>80%) should elute in the first 300 µl eluate. The remainder will elute in the second 300 µl. If dilution of the protein is undesirable, do not combine the eluates or, alternatively, elute in 100–200 µl aliquots.

Protocol: Protein Purification under Denaturing Conditions from *E. coli* Cell Lysates Using Ni-NTA Spin Columns

Materials and equipment to be supplied by user*

- Buffer A†: 6 M GuHCl, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl; pH 8.0
- Buffer B/7M urea: 7 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl; pH 8.0
- Buffer C: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl; pH 6.3
- Buffer D†: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl; pH 5.9
- Buffer E: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl; pH 4.5
- Benzonase® endonuclease 25 U/µl (e.g., Novagen cat. no. 70664-3)

Due to the dissociation of urea, the pH values of Buffers B, C, D, and E should be checked and, if necessary, adjusted immediately prior to use. Do not autoclave.

† Buffers A and D are not necessary for all proteins

Protocol

1. **Thaw cells for 15 min and resuspend in 700 µl Buffer B/7M urea and add 3 Units/ml culture volume Benzonase® Nuclease (i.e., for cell pellets from 5 ml cultures, add 15 Units Benzonase® Nuclease).**

   Cells from 5 ml culture are usually used, but culture volume used depends on protein expression level. Resuspending pellet in 700 µl buffer will allow recovery of a volume of cleared lysate of approx. 600 µl.

2. **Incubate cells with agitation for 15 min at room temperature.**

   The solution should become translucent when lysis is complete. Buffer B is the preferred lysis buffer, as the cell lysate can be analyzed directly by SDS-PAGE. If the cells or the protein do not solubilize in Buffer B, then Buffer A must be used. Since fractions which contain GuHCl will precipitate with SDS when loaded onto an SDS polyacrylamide gel, they must either be diluted (1:6), dialyzed before analysis, or separated from GuHCl by TCA precipitation (see Appendix C, page 54). Please note that Benzonase® Nuclease is inactive in the presence of GuHCl concentrations >100 mM. If cells are lysed in GuHCl, genomic DNA must be sedimented by centrifugation during collection of the cleared lysate supernatant as aggregated gDNA may clog the Ni-NTA spin column.

* 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.

3. **Centrifuge lysate at 12,000 x *g* for 15–30 min at RT (20–25°C) to pellet the cellular debris. Collect supernatant.**

   Save 20 µl of the cleared lysate for SDS-PAGE analysis.

4. **Equilibrate a Ni-NTA spin column with 600 µl Buffer B. Centrifuge for 2 min at 890 x *g* (approx. 2900 rpm).**

   The spin columns should be centrifuged with an open lid to ensure that the centrifugation step is completed after 2 min.

5. **Load up to 600 µl of the cleared lysate supernatant containing the His-tagged protein onto a pre-equilibrated Ni-NTA spin column. Centrifuge 5 min at 270 x *g* (approx. 1600 rpm), and collect the flow-through.**

   For proteins that are expressed at very high expression levels (50–60 mg of His-tagged protein per liter of cell culture) a 3x–5x concentrated cell lysate can be used. 600 µl of a 5x concentrated cell lysate in Buffer B will contain approximately 150–180 µg of His-tagged protein. For lower expression levels (1–5 mg/liter), 50 ml of cell culture should be used, to give a 50x concentrated cell lysate (600 µl cell lysate = 30–150 µg) of His-tagged protein.

   To ensure efficient binding, it is important not to exceed 270 x g (approx. 1600 rpm) when centrifuging Ni-NTA spin columns. At higher forces, even if the binding kinetics are high, the time the lysate is in contact with the resin is not sufficient for effective binding.

   Please take into account that the time needed for the centrifugation step during protein binding is influenced by the viscosity of the cleared lysate. For very concentrated cell lysates, it may be necessary to extend the centrifugation time to 3 or 4 min at 700 x g (approx. 2000 rpm).

   Save the flow-through for analysis by SDS-PAGE to check binding.

6. **Wash the Ni-NTA spin column with 600 µl Buffer C. Centrifuge for 2 min at 890 x *g* (approx. 2900 rpm).**

   This wash step can be carried out with Buffer C even if Buffer A was used to initially solubilize the protein. Most proteins will remain soluble in Buffer C. If this is not the case, Buffer C, Buffer D, and Buffer E should be made with 6 M guanidine hydrochloride instead of 8 M urea.

   Save the flow-through (wash fractions) for analysis by SDS-PAGE to check the stringency of the wash conditions.

7. **Repeat step 6.**

   It may not be necessary to wash twice with Buffer C. The number of wash steps required to obtain highly pure protein is determined primarily by the expression level of the His-tagged protein. When the expression level is high, 2 wash steps are usually sufficient for removal of contaminants. For very low expression levels or highly concentrated lysates, 3 wash steps may be required to achieve high purity.

   Save the flow-through (wash fractions) for analysis by SDS-PAGE to check the stringency of the wash conditions.

8. **Elute the protein twice with 200 µl Buffer E. Centrifuge for 2 min at 890 x *g* (approx. 2900 rpm), and collect the eluate.**

   Most of the His-tagged protein (>80%) should elute in the first 200 µl, especially when proteins smaller than 30 kDa are purified. The remainder will elute in the second 200 µl. If dilution of the protein is undesirable, do not combine the eluates or, alternatively, elute in 100–150 µl aliquots.

Protocol: Immunodetection of His-tagged Proteins with Penta·His Antibody (Chemiluminescent Method)

Of the two most commonly used immunodetection methods (chemiluminescent and chromogenic detection), chemiluminescence is the more sensitive. A protocol for chromogenic detection of His-tagged proteins and a comprehensive Troubleshooting Guide can be found in the *QIA*express *Detection and Assay Handbook*.

Materials and equipment to be supplied by user*

- Western blot
- TBS Buffer
- TBS-Tween/Triton Buffer
- Blocking buffer
- Anti-mouse secondary antibody conjugate. Either alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated anti-mouse IgG may be used. Rabbit-anti-mouse IgG/AP-conjugate from Pierce (Cat. No. 31332) or goat-anti-mouse IgG/HRP-conjugate from Jackson Immunoresearch (Cat. No. 115-035-003) yield good results.
- Secondary antibody dilution buffer

For chemiluminescent detection, BSA does not sufficiently block nonspecific binding of the secondary antibody to the membrane, and milk powder should be used to dilute the secondary antibody. Buffer containing milk powder should not be used for Penta·His Antibody dilution as this will reduce sensitivity. If alkali-soluble casein (Merck, Cat. No. 1.02241) is available in your country it can be used as a blocking reagent throughout the entire chemiluminescent detection protocol.

Chemiluminescent substrates

Please refer to manufacturer's recommendations. CDP-Star™ (e.g., from Applied Biosystems) can be used with AP-conjugated secondary antibodies, and the ECL™ system from Amersham Biosciences can be used in combination with HRP-conjugated secondary antibodies. The blocking reagents supplied with the CDP-Star system are compatible with Penta·His Antibodies and can be used, according to the manufacturer's instructions, instead of the blocking buffers and secondary antibody dilution buffers described in the following protocol (see Table 2).

* 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 2. Reagents used for chemiluminescent detection

Step	Reagent required
Blocking	3% BSA in TBS or 1% casein in TBS
Penta·His Antibody binding	3% BSA in TBS or 1% casein in TBS
Secondary antibody binding	10% milk powder in TBS or 1% casein in TBS

Procedure

1. **Wash membrane twice for 10 min each time with TBS buffer at room temperature (15–25°C).**
2. **Incubate for 1 h in blocking buffer at room temperature.**
   3% BSA (w/v) in TBS buffer*, is used for blocking until incubation.
3. **Wash membrane twice for 10 min each time in TBS-Tween/Triton buffer at room temperature (15–25°C).**
4. **Wash membrane for 10 min with TBS buffer at room temperature.**
5. **Incubate membrane in Penta·His Antibody solution (1/1000–1/2000 dilution of antibody or conjugate stock solution in blocking buffer) at room temperature (15–25°C) for 1 h.**
   Membrane can be sealed in plastic bags. Note: Do not use buffer containing milk powder for Penta·His Antibody dilution. This will reduce sensitivity. 3% BSA (w/v) in TBS buffer* is used for this blocking step when using chemiluminescent detection.
6. **Wash twice for 10 min each time in TBS-Tween/Triton buffer at room temperature (15–25°C).**
7. **Wash for 10 min in TBS buffer at room temperature (15–25°C).**
8. **Incubate with secondary antibody solution for 1 h at room temperature (15–25°C).**
   Dilute according to the manufacturer's recommendations. Use the lowest recommended concentration to avoid false signals. 10% nonfat dried milk in TBS* is used for incubation with secondary antibody when using chemiluminescent detection. Milk powder is needed to reduce background because BSA does not block sufficiently for the very sensitive chemiluminescent detection method.

* If alkali -soluble casein (Merck, Cat. No. 1.02241) is available in your country a 1% (w/v) solution in TBS buffer may be used for this protocol step.

9. **Wash 4 times for 10 min each time in TBS-Tween/Triton buffer at room temperature (15–25°C).**
10. **Perform chemiluminescent detection reaction and expose to X-ray film according to the manufacturer's recommendations.**

Troubleshooting: Expression

	Comments and suggestions
Poor or no cell growth	
a) Antibiotic not suitable	Check chosen antibiotic(s) and concentration(s). QIAgenes carry a kanamycin resistance gene. Add 50 µg/ml medium.
b) Protein to be expressed is toxic	Consider using *E. coli* strains carrying pLysS or pLysE to more tightly control expression. Express protein in a cell-free system.
No or low expression	
a) Protein is poorly expressed	Check that the protein is not found in the insoluble fraction. Move the His-tag to the C-terminus using the QIAgenes C-terminal His tag vector pQE-T7-2 (cat. no. 33023). It may not be possible to express the full-length protein in the given expression system, for example, due to toxicity or folding problems. Consider expressing truncations or an isolated domain of your protein of interest. Amplify the relevant coding sequence region by PCR and clone into pQE-T7-1 or -2 to express N- or C-terminally His-tagged protein (see Appendix D). Express protein in cell-free expression system.
b) Culture conditions for expression are incorrect	Use the same culture conditions and host cells to check the expression of TNFα encoded by the control plasmid.

		Comments and suggestions
c)	Protein is rapidly degraded	Perform a time course to check the kinetics of growth and induction. If the protein is small (<10 kDa), consider adding an N-terminal carrier protein such as GST or DHFR. If degradation occurs after cell lysis, consider adding protease inhibitors. Keep all samples and solutions at 4°C.
Inclusion bodies are formed		
a)	Expression level is too high (protein cannot fold correctly)	Reduce expression levels by modifying growth and induction conditions (see the section "High expression levels, insoluble proteins, and inclusion bodies" in *The QIA*expressionist).
b)	Protein is insoluble	Check both soluble and insoluble fractions for protein. Try to solubilize protein with denaturants or detergents. The QIA*expressionist* suggests ways to enhance solubility of proteins by altering the growth and induction conditions. **Note**: It may not be necessary to use denaturing conditions for purification if a small proportion of the protein of interest is insoluble or has formed inclusion bodies. Check the levels of soluble protein remaining in the cytoplasm that can be purified with Ni-NTA matrices.
c)	Protein is highly toxic	Consider using *E. coli* strains carrying pLysS or pLysE to more tightly control expression. Express protein in an in vitro system.

Troubleshooting: Purification

		Comments and suggestions
Protein does not bind to the Ni-NTA spin column		
a)	His tag is inaccessible	Purify protein under denaturing conditions. Move the His-tag to the C-terminus using the QIAgenes C-terminal His tag vector pQE-T7-2 (cat. no. 33023).
b)	His tag has been degraded	Check that the His tag is not associated with a portion of the protein that is processed.
c)	Binding conditions incorrect	Check pH of all buffers. Dissociation of urea often causes a shift in pH. The pH values should be checked immediately prior to use. Ensure that there are no chelating or reducing agents present and that the concentration of imidazole is not too high (see Table 1).
Protein elutes in the wash buffer		
a)	Wash stringency is too high	Lower the concentration of imidazole or increase the pH slightly.
b)	His tag is partially hidden	Purify under denaturing conditions.
c)	Buffer conditions incorrect	Check pH of denaturing wash buffer.
Protein precipitates during purification		
a)	Temperature is too low	Perform purification at room temperature.
b)	Protein forms aggregates	Try adding solubilization reagents such as glycerol (10–50%), 0.1% Triton® X-100 or Tween®-20, up to 20 mM β-ME, up to 2 M NaCl, or stabilizing cofactors such as Mg^{2+}. These may be necessary in all buffers to maintain protein solubility.

	Comments and suggestions
Protein does not elute	
Elution conditions are too mild (protein may be in an aggregate or multimer form)	Elute with decreased pH or increased imidazole concentration.
Protein elutes with contaminants	
a) Binding and wash conditions not stringent enough	Include 10–20 mM imidazole in the binding and wash buffers
b) Contaminants are truncated forms of the tagged protein	Check for possible internal translation starts (C-terminal tag) or premature termination sites (N-terminal tag). Prevent protein degradation during purification by working at 4°C or by including protease inhibitors.

References

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

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

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Appendix A: Buffer Compositions

Bacterial media and solutions

LB medium	10 g/liter tryptone; 5 g/liter yeast extract; 10 g/liter NaCl
LB agar	LB medium containing 15 g/liter agar
Psi broth	LB medium; 4 mM $MgSO_4$; 10 mM KCl
Kanamycin stock solution	10 mg/ml in H_2O, sterile filter, store in aliquots at –20°C
Chlormamphenicol stock solution	25 mg/ml in H_2O, sterile filter, store in aliquots at –20°C
IPTG (1 M)	238 mg/ml in H_2O, sterile filter, store in aliquots at –20°C

Buffers for preparing competent *E. coli*

TFB1	100 mM RbCl; 50 mM $MnCl_2$; 30 mM potassium acetate; 10 mM $CaCl_2$; 15% glycerol, adjust to pH 5.8*, sterile-filter
TFB2	10 mM MOPS; 10 mM RbCl; 75 mM $CaCl_2$; 15% glycerol, adjust to pH 6.8 with KOH, sterile filter

* Adjust pH carefully as insoluble Mn precipitates can form.

Buffers for purification under native conditions

NPI-10 (Binding/lysis buffer for native conditions, 1 liter)

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

Adjust pH to 8.0 using NaOH and sterile filter (0.2 or 0.45 µm).

NPI-20 (Wash buffer for native conditions, 1 Liter)

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

Adjust pH to 8.0 using NaOH and sterile filter (0.2 or 0.45 µm).

NPI-500 (Elution buffer for native conditions, 1 Liter)

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
500 mM Imidazole	34.0 g imidazole (MW 68.08 g/mol)

Adjust pH to 8.0 using NaOH and sterile filter (0.2 or 0.45 µm).

Buffers for purification under denaturing conditions

Buffer B/7 M urea (Denaturing lysis/binding buffer, 1 Liter)

7 M Urea	394.20 g urea (60.06 g/mol)
100 mM NaH_2PO_4	13.80 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
100 mM Tris·Cl	12.10 g Tris·Cl (MW 121.1 g/mol)

Adjust pH to 8.0 using HCl and sterile filter (0.2 or 0.45 µm).

Buffer C (Denaturing wash buffer, 1 liter)

8 M Urea	480.50 g urea (60.06 g/mol)
100 mM NaH_2PO_4	13.80 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
100 mM Tris·Cl	12.10 g Tris·Cl (MW 121.1 g/mol)

Adjust pH to 6.3 using HCl and sterile filter (0.2 or 0.45 µm).

Buffer E (Denaturing elution buffer, 1 liter)

8 M Urea	480.50 g urea (60.06 g/mol)
100 mM NaH_2PO_4	13.80 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
100 mM Tris·Cl	12.10 g Tris·Cl (MW 121.1 g/mol)

Adjust pH to 4.5 using HCl and sterile filter (0.2 or 0.45 µm).

PBS (1 liter)

50 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol)
150 mM NaCl	8.77 g NaCl (MW 58.44 g/mol)

Adjust pH to 7.2 using NaOH and sterile filter (0.2 or 0.45 µm).

SDS-PAGE sample buffers

2x SDS-PAGE sample buffer	0.09 M Tris·Cl pH 6.8; 20% glycerol; 2% SDS; 0.02% bromophenol blue; 0.1 M DTT
5x SDS-PAGE sample buffer	0.225 M Tris·Cl pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT

Solutions for SDS-PAGE

30% acrylamide/ 0.8% bis-acrylamide stock	30% acrylamide/0.8% bis-acrylamide (N,N'-methylene-bis-acrylamide) solution (e.g., Roth, Cat. No. 3029.1)
2.5x separating gel buffer	1.875 M Tris·Cl pH 8.9; 0.25% (w/v) SDS
5x stacking gel buffer	0.3 M Tris-phosphate pH 6.7; 0.5% (w/v) SDS
5x electrophoresis buffer	0.5 M Tris base; 1.92 M glycine; 0.5% (w/v) SDS. Should be pH 8.8. Do not adjust.

Solutions for western transfer

Semi-dry transfer buffer	25 mM Tris base; 150 mM glycine; 10% methanol. Should be pH 8.3 without adjusting.
Tank-blotting transfer buffer	25 mM Tris base; 150 mM glycine; 20% methanol. Should be pH 8.3 without adjusting.

Solutions for detection procedures

TBS buffer	10 mM Tris·Cl, pH 7.5; 150 mM NaCl
TBS-Tween buffer	20 mM Tris·Cl, pH 7.5; 500 mM NaCl; 0.05% (v/v) Tween 20 (Sigma, Cat. No. P1379)
TBS-Tween/Triton buffer	20 mM Tris·Cl, pH 7.5; 500 mM NaCl; 0.05% (v/v) Tween 20; 0.2% (v/v) Triton X-100 (Sigma, Cat. No. X-100)
Blocking buffers	3% (w/v) BSA (Sigma, Cat. No. A7906) in TBS buffer
	1% (w/v) alkali-soluble casein (Merck, cat. no. 1.02241) in TBS

Alkali-soluble casein is not easily dissolved in TBS. Dissolve casein and NaCl in 10 mM Tris base, and then adjust pH if necessary.

Secondary antibody dilution buffer for chemiluminescent detection	10% (w/v) skim milk powder (Fluka, cat. no. 70166) in TBS buffer. For best results, milk powder should be dissolved overnight at 4°C.

Coomassie staining solutions

Coomassie staining solution	0.05% (w/v) Coomassie Brilliant Blue R-250*; 40% (v/v) ethanol; 10% (v/v) glacial acetic acid. For 1 liter, dissolve 500 mg Coomassie Brilliant Blue R-250 in 400 ml 100% ethanol. Add 100 ml glacial acetic acid and water to 1 liter. Filter before use.
Destaining solution	40% (v/v) ethanol; 10% (v/v) glacial acetic acid

* e.g., SIGMA, cat. no. B 0149

Appendix B: Preparation of Competent *E. coli* Cells

Materials and equipment to be supplied by user*

- *E. coli* BL21 strain cells, e.g., BL21 (DE3), BL21 (DE3) pLysS
- LB agar plates containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid)
- LB medium containing 50 µg/ml kanamycin (and 25 µg/ml chloramphenicol if using *E. coli* BL21 cells containing the pLysS or pLysE plasmid)
- Buffers TFB1 and TFB2

For composition of media and solutions, see Appendix A, page 48.

Procedure

1. **Remove a trace of *E. coli* cells from their storage vial with a sterile toothpick or inoculating loop, and streak it out on LB agar containing the appropriate antibiotic(s).**
2. **Incubate at 37°C overnight.**
3. **Pick a single colony and inoculate 10 ml of LB medium containing the appropriate antibiotic(s). Grow overnight at 37°C.**
4. **Add 1 ml overnight culture to 100 ml prewarmed LB medium containing the appropriate antibiotic(s) in a 250 ml flask, and shake at 37°C until an OD_{600} of 0.5 is reached (approximately 90–120 min).**
5. **Cool the culture on ice for 5 min, and transfer the culture to a sterile, round-bottom centrifuge tube.**
6. **Collect the cells by centrifugation at low speed (5 min, 4000 x *g*, 4°C).**
7. **Discard the supernatant carefully. Always keep the cells on ice.**
8. **Resuspend the cells gently in cold (4°C) TFB1 buffer (30 ml for a 100 ml culture) and keep the suspension on ice for an additional 90 min.**
9. **Collect the cells by centrifugation (5 min, 4000 x *g*, 4°C).**
10. **Discard the supernatant carefully. Always keep the cells on ice.**
11. **Resuspend the cells carefully in 4 ml ice-cold TFB2 buffer.**

* 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.

12. **Prepare aliquots of 100–200 µl in sterile microcentrifuge tubes and freeze in liquid nitrogen or a dry-ice–ethanol mix. Store the competent cells at –70°C.**

    Competent cells frozen at –70°C can be stored for 6 months. Transformation efficiency should be tested using a control plasmid.

Appendix C: Preparation of Guanidine-Containing Samples for SDS-PAGE

Since the fractions that contain GuHCl will form a precipitate when treated with SDS, they must either be diluted with water (1:6), dialyzed before analysis, or separated from the guanidine hydrochloride by trichloroacetic acid (TCA) precipitation.

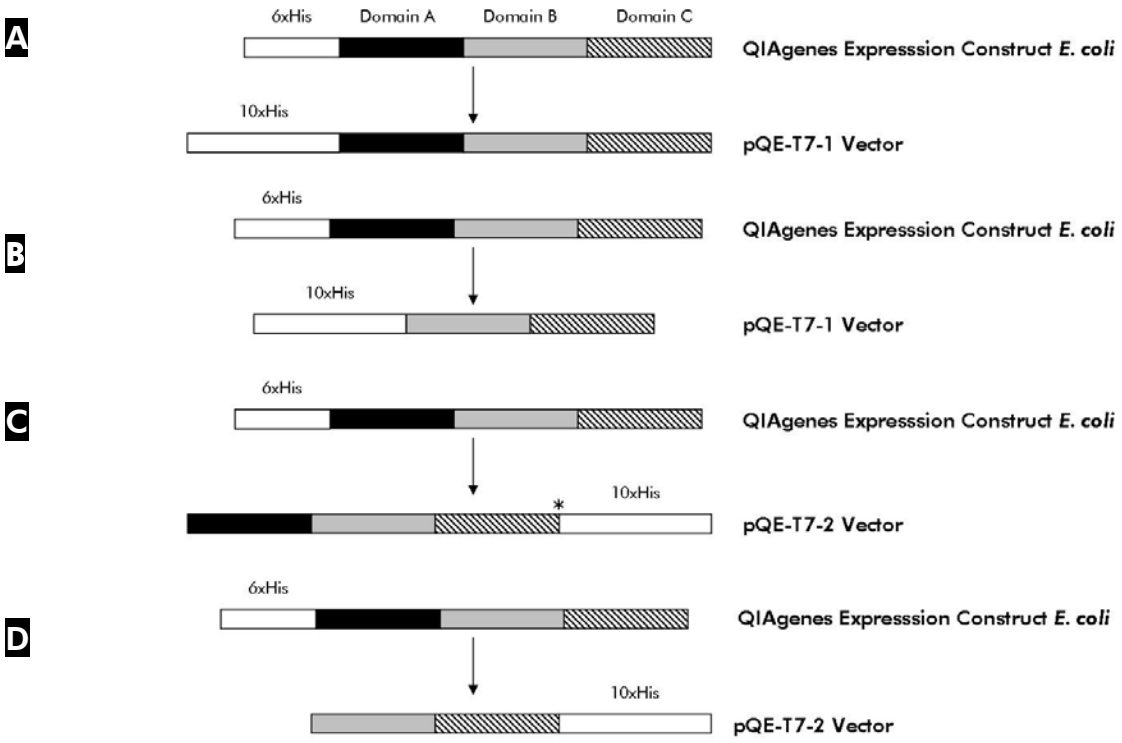
TCA-precipitation: Bring the volume of the samples up to 100 µl with water, add an equal volume of 10% TCA, leave on ice 20 min, spin 15 min at 15,000 x *g* in a microcentrifuge, wash pellet with 100 µl of ice-cold ethanol, dry, and resuspend in 1x SDS-PAGE sample buffer (5x SDS-PAGE sample buffer is 0.225 M Tris·Cl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT). In case there is still any GuHCl present, samples should be loaded immediately after boiling for 7 min at 95°C.

Appendix D: Cloning into QIAgenes N- and C-Terminal 10xHis tag vectors

Cloning vectors encoding N-terminal (pQE-T7-1) or C-terminal (pQE-T7-2) 10xHis tags are available for cloning of complete, truncated, or isolated domains of QIAgenes. The 10xHis tag is well-suited for expression of soluble proteins and especially useful for expression and purification of detergent-micelle–solubilized membrane proteins. Like QIAgenes Expression Constructs *E. coli*, both vectors provide a TAG amber translation stop which allows co-translational incorporation of a biotin label by the amber suppression principle in a cell-free expression reaction using the EasyXpress Site-Specific Labeling Kit (cat. no. 32602). The N-terminal His tag encoded by pQE-T7-1 can be cleaved off using TAGZyme exoproteases to create the native N-terminus, i.e., native sequence without any vector-encoded amino acids. The cloning strategies A–D listed in Figure 13 are described in detail on pages 57–63.

Protein coding sequences in QIAgenes Expression Constructs *E. coli* have been designed to avoid certain restriction enzyme recognition sites that may consequently be used for cloning. The following enzymes do not cut in any QIAgene Expression Construct *E. coli*:

*Sac*II, *Not*I, *Sac*I, *Kpn*I, *Pst*I, *Xho*I, and *Nde*I



* Additional amino acids (Leu-Glu) may be inserted between protein and tag, depending on strategy used.

Figure 13 Options for cloning QIAgenes Expression Construct sequences into pQE-T7-1 and pQE-T7-2 vectors. **A** Substituting a 6xHis tag with a 10xHis tag (see page 57); **B** Cloning of truncation or isolated domain with an N-terminal 10xHis tag (page 58); **C** Substituting an N-terminal 6xHis tag for a C-terminal 10xHis tag (page 59) or substituting an N-terminal 6xHis tag for a C-terminal 10xHis tag without extra amino acids inserted between protein and tag (page 61). ; **D** Subcloning a truncated version or a domain of a QIAgene with a C-terminal 10xHis tag and cloning it into pQE-T7-2 (page 62).

Strategy A: Cloning of QIAgenes into pQE-T7-1 for expression as N-terminally 10xHis-tagged proteins

An entire QIAgene sequence can be cloned into pQE-T7-1 by direct *Nde*I/*Xho*I cloning (see Figure 14).

Procedure

1. **Perform an *Nde*I/*Xho*I restriction of the QIAgene construct, load the cleavage reaction onto an agarose gel, and isolate the corresponding DNA fragment from the gel.**
2. **Perform an *Nde*I/*Xho*I restriction of the pQE-T7-1 vector, load the cleavage reaction onto an agarose gel, and isolate the DNA fragment corresponding to the linearized vector from the gel.**
3. **Ligate the QIAgene into the vector and transform the ligation reaction into *E. coli* (K12 strain).**
4. **Analyze transformants by a plasmid DNA miniprep and sequencing or by colony PCR using suitable primers.**

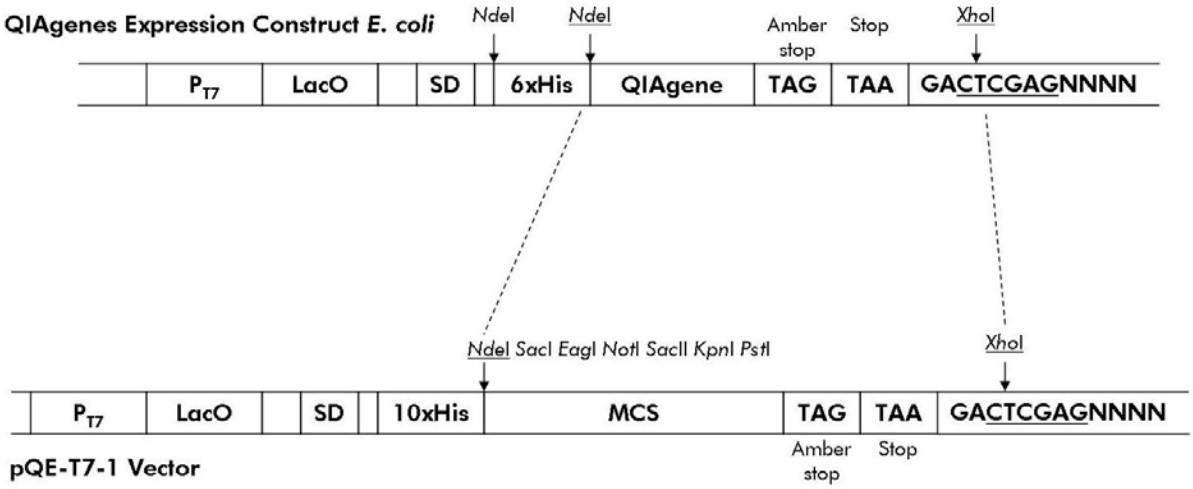


Figure 14. Cloning of a complete QIAgene sequence into pQE-T7-1. P_{T7}: T7 RNA polymerase promoter; **LacO**: Lac operator; **SD**: Shine-Dalgarno sequence (ribosomal binding site); **QIAgene**: target protein coding sequence.

Strategy B: Cloning a truncated version or a domain of a QIAgene by amplifying the corresponding DNA fragment via PCR and cloning it into pQE-T7-1

Procedure

1. **Design a 20 nucleotide sense PCR primer that hybridizes to the protein coding sequence. A non-hybridizing overhang is required with a 5' NdeI recognition sequence.**

   Apply standard primer design rules.

   Sense primer:

   5' GGAATTCCATATGAAACAGN$_{20}$ 3'

   where CATATG is the *Nde*I site, CAG is the TAGZyme stop, and N_{20} represents the first 20 desired N-terminal nucleotides of the desired truncation/domain coding sequence.

2. **Design an antisense primer that hybridizes to the protein coding sequence. A non-hybridizing overhang is required with a 5' XhoI restriction site, the TAA and TAG stop codons.**

   Apply standard primer design rules.

   Antisense primer:

   5' CCGCTCGAGTCTTACTAN$_{20}$ 3'

   where CTCGAG is the *Xho*I site, TTA is the second translation stop codon (TAA), CTA is the Amber stop codon (TAG), and N_{20} corresponds to the last 20 C-terminal nucleotides of the desired truncation/domain coding sequence.

3. **Amplify the QIAgene truncation/domain by proof-reading PCR, purify the PCR product, and perform an *Nde*I/*Xho*I restriction digest. Purify the the DNA fragment corresponding to the QIAgene from the restriction reaction.**
4. **Perform an *Nde*I/*Xho*I restriction digest of pQE-T7-1, load onto an agarose gel, and isolate the DNA band corresponding to the digested linearized vector from the gel.**
5. **Ligate the QIAgene into the vector and transform the ligation reaction into *E. coli* (K12 strain).**
6. **Analyze transformants by a plasmid DNA miniprep and sequencing or by colony PCR using the primers from steps 1 and 2.**

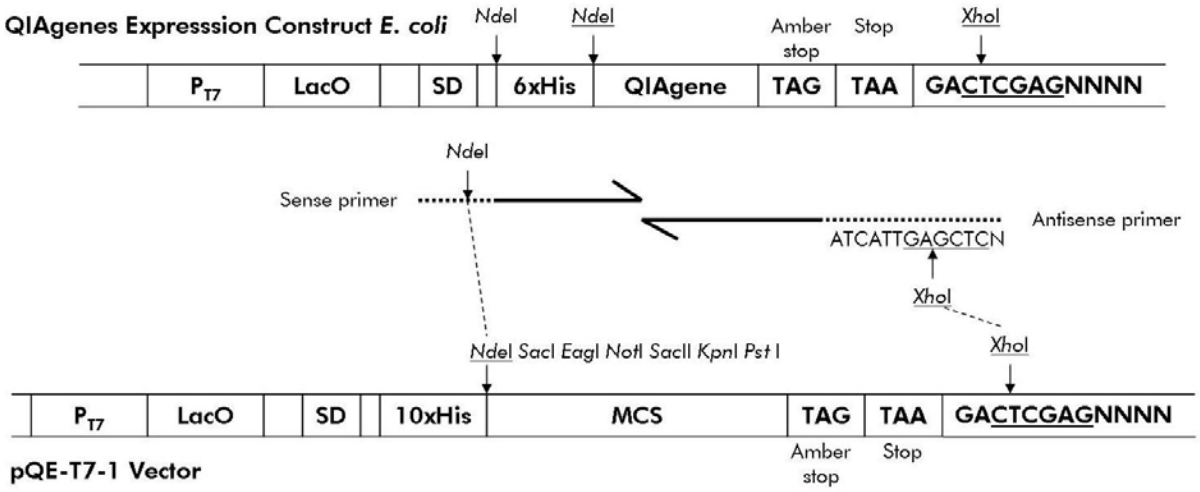


Figure 15. Cloning of a QIAgene sequence domain into pQE-T7-1. P_{T7}: T7 RNA polymerase promoter; **LacO**: Lac operator; **SD**: Shine-Dalgarno sequence (ribosomal binding site); **QIAgene**: target protein coding sequence.

Strategy C1: Cloning of QIAgenes into pQE-T7-2 for expression as C-terminally 10xHis-tagged proteins

A simple PCR strategy can be used to clone the entire QIAgene into pQE-T7-2 for expression as a C-terminally 10xHis-tagged protein with two additional amino acids (Leu–Glu; coded for by the *Xho*I recognition site CTCGAG) between the protein and the tag (see Figure 16). A second strategy (see page 61) can be used to create a construct for expressing proteins with C-terminal tags but without additional residues. However, this strategy requires the use of a long antisense primer.

Procedure

1. **Design a sense PCR primer that hybridizes to part of the N-terminal His tag of the QIAgene, the second *Nde*I site, and part of the protein coding sequence.**

   No non-hybridizing overhang is required. Apply standard primer design rules.

   Sense primer:

   5' CCATCACCATATGAAACAGN_{2-5} 3'

   where CCATCACCAT is part of the His tag, CATATG is the *Nde*I site, CAG is the TAGZyme stop, and N_{2-5} corresponds to the first 2–5 N-terminal nucleotides of the protein coding sequence.

2. **Design an antisense primer that hybridizes to the QIAgene construct sequences and includes a 5' *Xho*I site and part of the protein coding sequence.**

   A non-hybridizing overhang containing an *Xho*I site is required. Apply standard primer design rules.

   Antisense primer:

   5' CCGCTCGAGN_{20} 3'

   where CTCGAG is the *Xho*I site and N_{20} corresponds to the last 20 C-terminal nucleotides of the desired truncation/domain coding sequence.

3. **Amplify the QIAgene by proof-reading PCR, purify the PCR product, and perform an *Nde*I/*Xho*I restriction digest. Purify the DNA fragment corresponding to the QIAgene from the restriction reaction.**
4. **Perform an *Nde*I/*Xho*I restriction digest of pQE-T7-2, load onto an agarose gel, and isolate the DNA band corresponding to the digested linearized vector from the gel.**
5. **Ligate the QIAgene DNA fragment into the vector and transform the ligation reaction into *E. coli* (K12 strain).**
6. **Analyze transformants by a plasmid DNA miniprep and sequencing or by colony PCR using the primers from steps 1 and 2.**

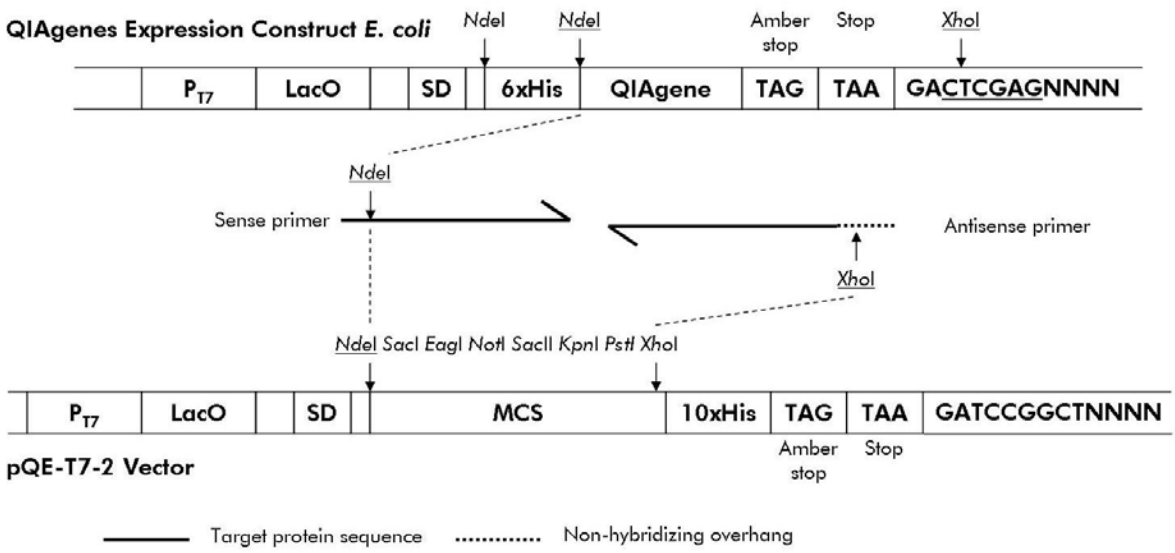


Figure 16. Cloning of a complete QIAgene sequence into pQE-T7-2. P_{T7}: T7 RNA polymerase promoter; **LacO**: Lac operator; **SD**: Shine-Dalgarno sequence (ribosomal binding site); **QIAgene**: target protein coding sequence.

Strategy C2: Creating a construct for expressing proteins with C-terminal tags but without additional residues.

Procedure

1. **Design a sense PCR primer that hybridizes to part of the N-terminal His tag of the QIAgene, the second *Nde*I site (underlined), and part of the protein coding sequence.**

   No non-hybridizing overhang is required. Apply standard primer design rules.

   Sense primer:

   5' CCATCACCATATGAAACAGN$_{2-5}$ 3'

   where CCATCACCAT is part of the His tag, CATATG is the *Nde*I site, CAG is the TAGZyme stop, and N_{2-5} corresponds to the first 2–5 N-terminal nucleotides of the protein coding sequence.

2. **Design an antisense primer that hybridizes to part of the protein coding sequence, and encodes a 5' *Xho*I site, TAA and TAG stop codons, and 10 histidine codons.**

   The 10xHis tag and stop codons encoded by the QIAgenes expression vector will not be used in this case.

   5' CCGCTCGAGTCTTACTAATGATGATGGTGGTGATGGTGATGATGATGN$_{20}$3'

   where CTCGAG is the *Xho*I site, TTA is the second translation stop (TAA), CTA is the Amber stop (TAG), ATGATGATGGTGGTGATGGTGATGATGATG is the 10xHis tag, and N_{20} corresponds to the last 20 C-terminal nucleotides of the protein coding sequence.

3. **Amplify the QIAgene by proof-reading PCR, purify the PCR product, and perform an *Nde*I/*Xho*I restriction digest. Purify the DNA fragment corresponding to the QIAgene from the restriction reaction.**
4. **Perform an *Nde*I/*Xho*I restriction digest of pQE-T7-2, load onto an agarose gel, and isolate the DNA band corresponding to the digested linearized vector from the gel.**
5. **Ligate the QIAgene DNA fragment into the vector and transform the ligation reaction into *E. coli* (K12 strain).**
6. **Analyze transformants by a plasmid DNA miniprep and sequencing or by colony PCR using the primers from steps 1 and 2.**

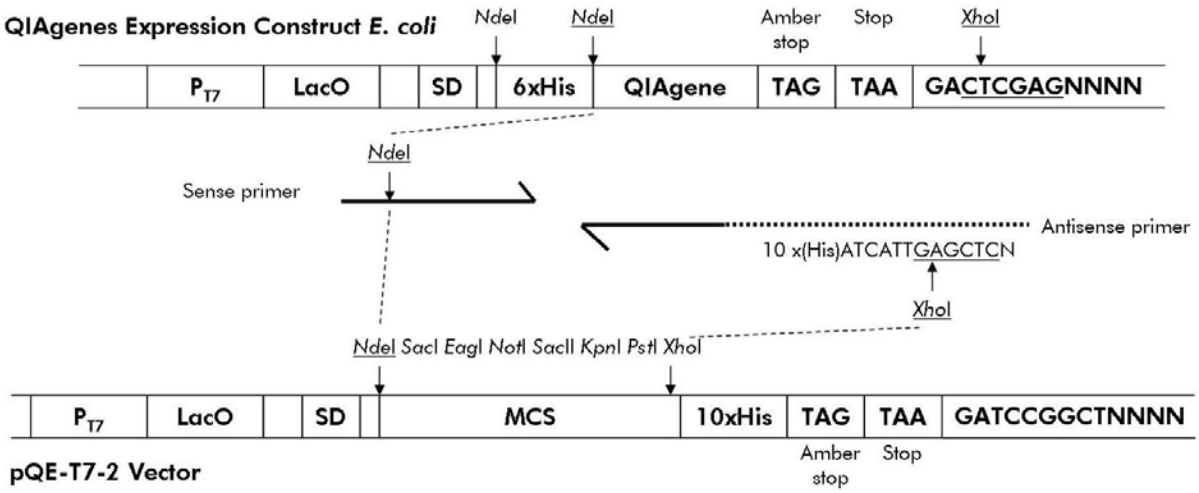


Figure 17. Cloning of a complete QIAgene sequence into pQE-T7-2 without extra amino acids between the native sequence and the tag. P_{T7}: T7 RNA polymerase promoter; **LacO**: Lac operator; **SD**: Shine-Dalgarno sequence (ribosomal binding site); **QIAgene**: target protein coding sequence.

Strategy D: Subcloning a truncated version or a domain of a QIAgene with a C-terminal 10xHis tag and cloning it into pQE-T7-2

Procedure

1. **Design a sense PCR primer that contains a 5' *Nde*I site and hybridizes to part of the QIAgene protein coding sequence.**

   Apply standard primer design rules.

   Sense primer:

   5' GGAATTCCATATGAAAN$_{20}$ 3'

   where CATATG is the *Nde*I site and N_{20} corresponds to the first 20 "N-terminal" nucleotides of the desired truncation/domain protein coding sequence.

2. **Design an antisense primer that hybridizes to part of the protein coding sequence, and encodes a 5' *Xho*I site (see alternative strategy below to avoid introduction of extra amino acids).**

   A non-hybridizing overhang containing an *Xho*I site is required. Apply standard design rules.

   Antisense primer:

   5' CCGCTCGAGN$_{20}$ 3'

   where CTCGAG is the *Xho*I site and N_{20} corresponds to the last 20 "C-terminal" nucleotides of the desired truncation/domain coding sequence. This strategy makes use of the 10xHis tag encoded by the vector and

introduces the two additional amino acids Leu-Glu (encoded by the *Xho*I site) between the QIAgene domain and the His tag.

Alternative strategy avoiding introduction of additional amino acids (see Figure 18):

Antisense primer:

5' CCGCTCGAGTCTTACTAATGATGATGGTGGTGATGGTGATGATGATGN203'

where CTCGAG is the *Xho*I site, TTA is the second translation stop (TAA), CTA is the Amber stop (TAG), ATGATGATGGTGGTGATGGTGATGATGATG codes for a 10xHis tag, and N_{20} corresponds to the last 20 "C-terminal" nucleotides of the desired truncation/domain coding sequence. The 10xHis tag and stop codons encoded by the QIAgenes expression vector will not be used in this case.

3. **Amplify the QIAgene by proof-reading PCR, purify the PCR product, and perform an *Nde*I/*Xho*I restriction digest. Purify the DNA fragment corresponding to the QIAgene from the restriction reaction.**
4. **Perform an *Nde*I/*Xho*I restriction digest of pQE-T7-2, load onto an agarose gel, and isolate the DNA band corresponding to the digested linearized vector from the gel.**
5. **Ligate the QIAgene DNA fragment into the vector and transform the ligation reaction into *E. coli* (K12 strain).**
6. **Analyze transformants by a plasmid DNA miniprep and sequencing or by colony PCR using the primers from steps 1 and 2.**

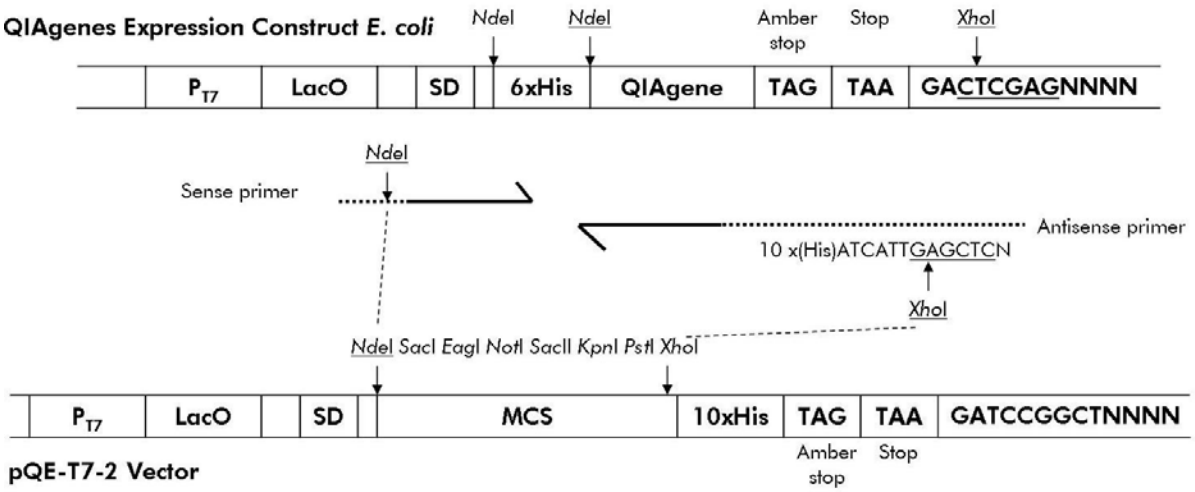


Figure 18. Cloning of a N-terminally truncated QIAgene sequence into pQE-T7-2 without extra amino acids between the native sequence and the tag. P_{T7}: T7 RNA polymerase promoter; **LacO**: Lac operator; **SD**: Shine-Dalgarno sequence (ribosomal binding site); **QIAgene**: target protein coding sequence.

Ordering Information

Product	Contents	Cat. no.
QIAgenes Expression Kit	QIAgenes Expression Construct (10 µg), QIAgenes *E. coli* Positive Control (10 µg), Penta·His Antibody, BSA-free (3 µg), 4 Ni-NTA Spin Columns	Varies
QIAgenes N-terminal His tag Vector pQE-T7-1	25 µg vector DNA	33013
QIAgenes C-terminal His tag Vector pQE-T7-2	25 µg vector DNA	33023
Related products		
Ni-NTA Spin Kit (50)	50 Ni-NTA Spin Columns, Reagents, Buffers, Collection Tubes, 1 µg Control Expression Plasmid	31314
Ni-NTA Spin Columns (50)	50 Ni-NTA Spin Columns, Collection Tubes	31014
Penta·His Antibody, BSA-free (100 µg)	100 µg mouse anti-$(His)_5$ (lyophilized, BSA-free, for 1000 ml working solution)	34660
Ni-NTA Superflow Cartridges (5 x 1 ml)	5 cartridges pre-filled with 1 ml Ni-NTA Superflow: for automated purification of His-tagged proteins using liquid chromatography systems	30721
Ni-NTA Superflow Cartridges (100 x 5 ml)	100 cartridges pre-filled with 5 ml Ni-NTA Superflow: for automated purification of His-tagged proteins using liquid chromatography systems	30765
pQE vectors — for high-level expression of recombinant proteins carrying His tags		
C-Terminus pQE Vector Set	25 µg each: pQE-16, pQE-60, pQE-70	32903
N-Terminus pQE Vector Set	25 µg each: pQE-9, pQE-30, pQE-31, pQE-32, pQE-40	32915
cis-Repressed pQE Vector Set	25 µg each: pQE-80L, pQE-81L, pQE-82L	32923

Product	Contents	Cat. no.
pQE-100 DoubleTag	25 µg pQE-100 (lyophilized) Vector DNA	33003
pQE-30 Xa Vector	25 µg pQE-30 Xa Vector DNA	33203
pQE-TriSystem Vector	25 µg pQE-TriSystem Vector DNA	33903
QIAexpress UA Cloning Kit	100 µl 2x Ligation Master Mix, 1 µg pQE-30 UA Vector DNA (50 ng/µl), distilled water	32179
His·*Strep* pQE-TriSystem Vector Set	pQE-TriSystem His·*Strep* 1 and pQE-TriSystem His·*Strep* 2 vectors, 25 µg each	32942
TAGZyme pQE Vector Set	TAGZyme pQE-1 and pQE-2 Vector DNA, 25 µg each	32932
EasyXpress kits — for cell-free synthesis of recombinant proteins		
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 Insect Kit II (5)	For 5 x 50 µl reactions: *Spodoptera frugiperda* insect cell extract, reaction buffers, in vitro transcription reaction components, RNase-free water, gel-filtration columns, and positive-control DNA	32561
EasyXpress Protein Synthesis Mini Kit	For 20 x 50 µl reactions: *E. coli* extract, reaction buffer, RNase-free water, and positive-control DNA	32502
EasyXpress Protein Synthesis Maxi Kit	For reactions up to 4000 µl: 4 x 350 µl *E. coli* extract, reaction buffer, RNase-free water, and positive-control DNA	32506
EasyXpress 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 Random Biotin Kit	For 60 x 50 µl reactions: 4 x 15 µl EasyXpress Biotinyl-Lysyl tRNA (Phe)	32612

Product	Contents	Cat. no.
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
EasyXpress NMR Protein Synthesis Kit – X	For 2 x 5 ml reactions: *E. coli* extract, reaction buffers, amino acid mix w/o amino acid X (to be supplied by user), RNase-free water, gel-filtration columns, and reaction flasks	Varies
Anti·His antibodies and conjugates — for sensitive and specific detection of His-tagged proteins		
RGS·His Antibody (100 µg)	100 µg mouse anti-RGS(His)$_4$ (lyophilized, with BSA, for 1000 ml working solution)	34610
RGS·His Antibody, BSA-free, (100 µg)	100 µg mouse anti-RGS(His)$_4$ BSA-free (lyophilized, for 1000 ml working solution)	34650
Penta·His Antibody, BSA-free (100 µg)	100 µg mouse anti-(His)$_5$ (lyophilized, BSA-free, for 1000 ml working solution)	34660
Tetra·His Antibody, BSA-free (100 µg)	100 µg mouse anti-(His)$_4$ (lyophilized, BSA-free, for 1000 ml working solution)	34670
Anti·His Antibody Selector Kit	RGS·His Antibody, Penta·His Antibody, Tetra·His Antibody, all BSA-free, 3 µg each	34698
RGS·His HRP Conjugate Kit	125 µl RGS·His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer, 10x Concentrate	34450
Penta·His HRP Conjugate Kit	125 µl Penta·His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer, 10x Concentrate	34460
Tetra·His HRP Conjugate Kit	125 µl Tetra·His HRP Conjugate, 5 g Blocking Reagent, 50 ml Blocking Reagent Buffer, 10x Concentrate	34470

Product	Contents	Cat. no.
His Protein Ladder	His-tagged marker proteins (lyophilized, for 50–100 lanes on western blots)	34705
His-tag removal systems		
TAGZyme Kit	For processing of approximately 10 mg tagged protein: 0.5 units DAPase Enzyme, 30 units Qcyclase Enzyme, 10 units pGAPase Enzyme, 20 mM Cysteamine·HCl (1 ml), Ni-NTA Agarose (10 ml), 20 Disposable Columns	34300

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