Expression in *E. coli*

Expression of recombinant proteins can be approached in general by starting with a plasmid that encodes the desired protein, introducing the plasmid into the required host cell, growing the host cells and inducing expression, and ending with cell lysis and SDS-PAGE analysis to verify the presence of the protein (Figure 16). With careful choice of host strains, vectors, and growth conditions, most recombinant proteins can be cloned and expressed at high levels in *E. coli*. However, many polypeptide gene products expressed in *E. coli* accumulate as insoluble aggregates that lack functional activity. Other problems with protein expression may include cell toxicity, protein instability, improper processing or post-translational modification, and inefficient translation.

**Figure 16.** Expression of 6xHis-tagged proteins with the QIAexpression System.
A primary consideration for recombinant protein expression and purification is the experimental purpose for which the protein will be utilized. For biochemical and structural studies, it is often important to optimize conditions for the expression of soluble, functionally active protein, whereas for antigen production, the protein can be expressed either in native or denatured form. The QIAexpress System is optimized for high expression levels, but conditions for optimal expression of individual proteins must be determined empirically. Freshly transformed bacterial colonies often express recombinant proteins at different levels. Therefore, comparison of the signals produced after colony blotting to identify high-expressing colonies can help significantly while establishing expression cultures.

Optimal growth and expression conditions for the protein of interest should be established with small-scale cultures before large-scale protein purification is attempted. In order to judge the toxicity of an expressed protein, cell growth before and especially after induction of expression should be monitored. Expression of the nontoxic, 26 kDa DHFR protein encoded by the control plasmid pQE-40 is an ideal control for cell growth, expression, and purification. A much slower growth rate of the host cells expressing the protein of interest suggests that the gene product may be toxic to the E. coli host. Conversely, a much faster growth rate may indicate inefficient transcription or translation due to plasmid instability, inefficient induction, or deletions in the control region.

**Note:** Control plasmid pQE-40 encoding the mouse DHFR is optimized for high-level expression producing up to 40 mg/liter in culture. Since the protein accumulates to almost 90% in an insoluble form in inclusion bodies, purification under denaturing conditions in the presence of urea is recommended. However, approximately 10% of the DHFR protein remains in a soluble form that can be purified under native conditions. The recombinant DHFR protein migrates at 26 kDa on SDS-PAGE gels.

Many factors may contribute to difficulties encountered when expressing foreign proteins in E. coli. The following sections address these difficulties in more detail.

**Basic principles**

**Culture media**

The media of choice for the growth of M15 cells containing a pQE expression plasmid and the pREP4 repressor plasmid are LB medium and its modifications, 2x YT, or Super Broth, each containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Initially it is advisable to try expression in all three 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 noted. Using the cis-repressed vectors pQE-80L, pQE-81L, or pQE-82L without pREP4 kanamycin should not be included in the growth medium.
**Maintenance of the expression plasmid**

Poor plasmid maintenance in the cells can lead to low expression levels. Ampicillin is an unstable antibiotic and is rapidly depleted in growing cultures due in part to the \(\beta\)-lactamase secreted by resistant bacterial cells. It is important to check plasmid levels by plating cells from the expression culture on plates with and without ampicillin. If the stability of the expression construct is a problem, the cultures should be grown in the presence of 200 \(\mu\)g/ml ampicillin, and the level should be maintained by supplementing ampicillin during long growth periods. Alternatively, the cultures may be grown in the presence of carbenicillin, a more stable \(\beta\)-lactam, at 50 \(\mu\)g/ml.

Host cells containing the pREP4 repressor plasmid should be maintained in the presence of kanamycin at 25 \(\mu\)g/ml. Many of the *E. coli* host strains that contain the lacI\(^{q}\) mutation harbor it on an F-factor (see also “Propagation of pQE plasmids and constructs”, page 35). Therefore these strains should be checked for the presence of the F-factor before they are transformed with the pQE vectors. For example, XL1-Blue strains can be selected on tetracycline.

**Small-scale expression cultures**

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. The use of small expression cultures, and the preparation of lysates followed by purification by Ni-NTA affinity chromatography, provide a rapid way to judge the effects of varied growth conditions on expression levels and solubility of recombinant proteins. Expression levels vary between different colonies of freshly transformed cells, and small-scale preparations permit the selection of clones featuring optimal expression rates (see Protocol 5, page 45).

**Time-course analysis of protein expression**

To optimize the expression of a given protein construct, a time-course analysis of the level of protein expression is recommended (Figure 17). 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 6xHis-tagged protein present at various times after induction in the soluble and insoluble fractions (see Protocol 14, page 85 and Protocol 19, page 92), the optimal induction period can be established.

A protocol using Ni-NTA spin columns is provided on page 45.
Figure 17. Time course of expression using the QIAexpress System. Expression of 6xHis-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: noninduced control.

Specific considerations

Low expression levels

Low-level expression can occur because the protein is toxic or unstable, or because the expression construct is not maintained in the cells during growth. In some cases, the 5' end of the inserted DNA sequence may encode elements that interfere with transcription or translation (e.g., masking of the Shine-Dalgarno sequence by stem-loop structures resulting from inverted repeats); in these instances the sequence being expressed should be checked and modified if necessary. Modifications of growth media and different host strains may also have an effect on expression.

Toxic gene products

The expression of heterologous proteins can slow the growth of the host cells. High transcription rates lead to slow growth, and this in turn is compounded by metabolic demands imposed by translation of the recombinant protein. Gene products that affect the host cell’s growth rate at low concentrations are considered to be toxic; examples include membrane proteins or proteins that interact with DNA or interfere with electron transport.

Some proteins are only mildly toxic and appear to be expressed poorly after the cells have been kept in culture media or on plates for several days. The maintenance of cells can be complicated by the fact that the desired cells are likely to be outgrown by cells that harbor mutants of the plasmid and do not express the toxic protein. Constructs encoding proteins
that are more toxic generally lower the transformation efficiencies of the host cells as compared to the original parent vector, and plasmids in transformants that do arise often have deletions and mutations.

To reduce the effects of protein toxicity on cell growth prior to induction, the level of basal transcription that occurs in the absence of induction ("leakiness") should be repressed as much as possible, and the number of generations before induction should be kept to a minimum. Problems related to the loss of plasmids can sometimes be overcome by growing the cells in the presence of high levels of ampicillin (200 µg/ml) or carbenicillin (50 µg/ml). For instable expression constructs, overnight starter cultures should be avoided. Colonies from a fresh plate should be inoculated into a small starter culture and grown for 2–3 hours, until mid-log phase. This starter culture should then be diluted 20–50-fold in prewarmed medium and grown to an OD$_{600}$ of approximately 0.5 before induction.

For very toxic proteins, we recommend using the pQE-80L series of expression vectors in the M15[pREP4] host strain. This combination of two repressor modules results in highly efficient suppression of recombinant protein expression prior to induction and gives the best chance of successful expression of toxic proteins.

**Hydrophobic regions**

Recombinant proteins with hydrophobic regions often have a toxic effect on host cells, most likely due to the association of the protein with or incorporation into vital membrane systems. Sequences encoding signal peptides or transmembrane domains, unless of specific interest, should be removed from the DNA inserts before they are cloned. However, it is also possible to express proteins containing signal peptides that target the protein molecules into the periplasmic space, albeit at a lower rate (see also "Secretion", page 23). Lowering the growth temperature to 25°C before induction is recommended. Examples of 6xHis-tagged, transmembrane proteins with membrane-spanning domains that have been expressed to significant levels in *E. coli* have also been reported (Waeber *et al.* 1993).

**Unstable proteins**

Some proteins, particularly those that are smaller than 10 kDa, are not stable in *E. coli*, and may be degraded rapidly by proteases. This may be overcome by:

- Reducing the growth temperature to 30°C
- Inducing for a shorter period of time
- Using a host strain deficient in one or more proteases (e.g. OmpT, Lon$^-$)
- Expressing short proteins and peptides as a fusion with DHFR (plasmids pQE-16 and pQE-40)
- Including glycerol in the purification buffers

If the protein is degraded during the purification process, it may be necessary to use one or more protease inhibitor, such as PMSF, leupeptin, or aprotinin (Wingfield 1995a) and to work at 4°C all times.
High expression levels, insoluble proteins, and inclusion bodies

Eukaryotic proteins expressed intracellularly in *E. coli* are frequently sequestered into insoluble inclusion bodies. The intermolecular association of hydrophobic domains during folding is believed to play a role in the formation of inclusion bodies. For proteins with cysteine residues, improper formation of disulphide bonds in the reducing environment of the *E. coli* cytoplasm may also contribute to incorrect folding and formation of inclusion bodies.

An advantage of the QIAexpress purification system is that 6xHis-tagged proteins in insoluble inclusion bodies can be easily solubilized with denaturants such as 6 M Gu-HCl or 8 M urea or with a variety of detergents and be purified on Ni-NTA matrices. Proteins purified under denaturing conditions can then be refolded if necessary before use (Wingfield and Palmer 1995). Some general tips for the protein refolding are given on page 106 in the section “Protein refolding recommendations”.

While it is possible to obtain functionally active protein using this approach, many researchers have found that their recoveries are poor when a refolding step is included in the purification protocol. An alternative approach is to adjust expression conditions such that smaller amounts of recombinant protein are produced in a soluble, native form. Even if inclusion bodies are formed, some 6xHis-tagged protein will often remain soluble in the cytoplasm, from which they can be purified on Ni-NTA matrices under native conditions (see protocols beginning on page 82). If higher levels of soluble protein are desired, a reduction in growth temperature following induction may be helpful. Growth temperature often directly affects both expression levels and protein solubility, and lower temperatures will reduce expression levels leading to a higher amount of soluble protein. Alternatively, the culture can also be grown to a higher cell density before induction and the expression period can be kept to a minimum. The IPTG concentration can be reduced from 1 mM to 0.005 mM, which would reduce the expression level by 90–95%. Furthermore, it may be sufficient to change the host strain used, since certain strains tolerate some proteins better than others and allow higher levels of expression before forming inclusion bodies. Finally, many proteins require metal cofactors in order to remain soluble, and the addition of metal salts to the culture media may be helpful. If the metal requirements of the protein are not known, a number of different supplements should be tested. Note that some divalent cations may interfere with protein binding to Ni-NTA.

Other Expression Systems

The choice of expression system is dictated by the specific applications for which the protein is being produced. Bacterial expression is recommended for obtaining maximum expression rates and if post-translational modifications of the recombinant protein are not required for protein function. After initial studies that can be carried out in bacterial expression systems, expression in another system such as insect, yeast, or mammalian cells may be required in order to obtain higher protein activity, functional disulfide bridges, or eukaryotic-specific post-translational modifications.
Expression

Baculovirus

Baculovirus vectors used to heterologously express proteins in insect cells have also become widely used (Luckow 1991; Miller 1988; O’Reilly et al. 1994). These systems are based on the ability of the baculovirus to infect and multiply in cultured insect cells. The most widely used virus is the Autographa californica nuclear polyhedrosis virus (AcNPV), a lytic virus that infects lepidopterans. A foreign gene is cloned into a plasmid transfer vector and then cotransfected along with double-stranded baculovirus DNA into insect cells. Homologous recombination of the plasmid and insert DNA with viral DNA in vivo leads to the insertion of the sequence encoding the recombinant protein into the viral genome. Insect cells which produce the recombinant proteins also recognize most vertebrate protein-targeting sequences and can thus express a wide variety of proteins, including cytoplasmic, nuclear, membrane-spanning, and secreted proteins. Many post-translational modifications typically encountered in vertebrate cells, such as phosphorylation, glycosylation, precursor processing, and targeting are also carried out in insect cells. Recombinant proteins can thus be either produced within the cells or secreted into the culture medium.

The pQE-TriSystem vector is a suitable shuttle vector for recombinant protein expression in insect cells. It carries two segments of the AcNPV genomic DNA (ORF 603 and ORF 1629) bracketing the p10 promoter, a multiple cloning site and transcription-terminator sequences (see vector information page 56, and vector map below). Due to the presence of the AcNPV sequences, recombinant baculovirus can be generated by homologous recombination at the polh locus. Generally, recombinant baculoviruses are prepared by cotransfection of the shuttle vector and linearized baculovirus genomic DNA into insect cells such as Sf9 and Sf21 cells. The pQE-TriSystem vector is compatible with the genomic baculovirus preparations derived from Autographa californica polyhedrosis virus (AcNPV) commercially available as BacVector®-3000 Triple Cut Virus DNA (Novagen, cat. no. 70078-3), BaculoGold™ linearized baculovirus DNA (BD Pharmingen, cat. no. 21100D) and BacPAK6 DNA (Clontech, cat. no. 6144-1). Optimized protocols for cotransfection, virus amplification and plaque assay for virus titer determination can be taken from the protocols of the different suppliers.

Several established cell lines are highly susceptible to AcNPV virus infection. The two most frequently used insect cell lines are Sf9 and Sf21 established from ovarian tissues of Spodoptera frugiperda larvae. Expression levels of recombinant protein vary between 0.05–50% of the total insect-cell protein content. For optimal protein production the multiplicity of infection (MOI, number of applied viruses per cell) should be between 0.2 and 10. Researchers should test different MOI to empirically determine optimum levels of protein production. With increasing MOI, protein production and subsequent cell lysis will be accelerated. Use of a MOI higher than one means that a considerable portion of the virus population may contain deletion mutants or non-recombinant viruses leading to reduced expression levels of the correct recombinant protein.
Heterologous gene-expression levels in the baculovirus system can vary by approximately 1000-fold depending on the intrinsic nature of the gene and the encoded protein. Optimization of the gene construct will generally influence protein production by only 2–5 fold. Two main factors should be considered. Firstly, the 5’-untranslated region between the promoter and the ATG start codon should be reduced in length, and secondly, additional ATG codons upstream of the gene should be avoided, since translation will start at the first ATG initiation codon downstream of the promoter. In the pQE-TriSystem vector the distance between the promoter and ATG start codon in front of the multiple cloning site is optimized for recombinant gene expression.

A protocol for the purification of 6xHis-tagged proteins from baculovirus-infected insect cells appears on page 88 of this handbook.

**Figure 18.** pQE-TriSystem vector for parallel protein expression using a single construct in E. coli, insect, and mammalian cells. PT5: T5 promoter, lac O: lac operator, RBS: ribosome binding site, ATG: start codon, 8xHis: His tag sequence, MCS: multiple cloning site, Stop Codons: stop codons in all three reading frames, Ampicillin: ampicillin resistance gene, P CAG: CMV/actin/globin promoter, P p10: p10 promoter, Kozak: Kozak consensus sequence, termination region: transcription terminator region, lef2, 603/1629: flanking baculovirus sequences to permit generation of recombinant baculoviruses, pUC: pUC origin of replication.
Figure 19. pQE-TriSystem promoter region overview and sequencing primer annealing positions

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Mammalian cells

Even though expression levels are usually low, mammalian cells are often the best host for the expression of recombinant vertebrate proteins because they produce the same post-translational modifications and recognize the same signals for synthesis, processing, and secretion utilized in the organism from which the sequence was originally derived. A wide variety of mammalian expression vectors are currently in use. In general they contain an efficient promoter element for high-level transcription initiation, mRNA processing signals such as mRNA cleavage and polyadenylation sequences, selectable markers to select mammalian cells that have stably integrated the DNA into their genome, and plasmid sequences that permit the propagation of the vectors in bacterial hosts.

pQE-TriSystem is a suitable vector for transient recombinant protein expression in mammalian cells. The combination of the CMV immediate-early enhancer fused to the chicken β-actin promoter results in a strong promoter for constitutive heterologous gene expression. The polyadenylation signals of the mRNA transcript are encoded by the downstream rabbit-globin terminator. The presence of the Kozak consensus sequence including the ATG start codon facilitates efficient translation initiation. pQE-TriSystem vector can be introduced into the cell by traditional transfection techniques such as calcium phosphate or liposome mediated transfection, and electroporation. QIAGEN offers three transfection reagents based on the latest advances in transfection technology; the non-liposomal—lipid—based Effectene™ Transfection Reagent, and the proven activated-dendrimer—based Superfect® Transfection Reagent and Polyfect® Transfection Reagent. A protocol for purification of 6xHis tagged proteins from mammalian cells is given on page 86.

Yeast

Expression of recombinant proteins in yeast combines the advantages of providing most eukaryotic post translational modifications such as phosphorylation, glycosylation, and targeting, with expression levels ranging up to several milligrams per liter of culture (up to 30% of the expression of total yeast protein). A comprehensive review is provided by Romanos and coworkers (1992).

The most widely used expression vectors are E. coli/yeast shuttle plasmids (Baldrarini and Cesareni 1985; Clare et al. 1991) that are mitotically stabilized by autonomously replicating sequences (ARS/CEN region, 2µ locus) or by integration into the yeast genome. The episomal expression constructs are introduced into the cells by transformation into competent cells (Gietz et al. 1992) or by electroporation. Various strategies can be employed to induce expression of the recombinant proteins encoded by these constructs, but the specific method chosen depends on the physiological characteristics of the yeast strain being used. Commonly used strategies are exemplified by the galactose-inducible expression systems in Saccharomyces cerevisiae and methanol-driven induction in the methylotrophic yeast, Pichia pastoris. Intracellular 6xHis-tagged proteins to be purified must be released from the cells by disrupting cell walls by enzymatic, chemical, or mechanical
means (Sambrook et al. 1989; Ausubel et al. 1995; Guthrie and Fink 1991). Purification can be simplified by including an export signal in-frame with the 6xHis tag. Proteins will be targeted into the medium and can then be purified by Ni-NTA affinity chromatography. Yeast cells may acidify the culture medium and both the cells and the medium may contain certain compounds that influence binding of 6xHis tags to Ni-NTA matrices. Methods used to remove these ingredients and purify the 6xHis-tagged proteins are described in the section “Purification of 6xHis-tagged proteins produced in other expression systems” on page 76.
Expression procedures

Protocol 6. Determination of target protein solubility

Materials
LB medium
Kanamycin stock solution
Ampicillin stock solution
Lysis buffer for purification under native conditions
1x PAGE sample buffer
2x PAGE sample buffer
Buffer compositions are provided in the appendix on page 111.

Culture growth
1. Inoculate 10 ml LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin in a 50 ml flask. Grow the cultures overnight at 37°C with shaking.
   Kanamycin should be omitted when using the cis-repressed pQE-80L series of vectors.
2. Inoculate 50 ml of prewarmed media (with antibiotics) with 2.5 ml of the overnight cultures and grow at 37°C, with vigorous shaking (~300 rpm), until the OD 600 is 0.5–0.7 (approximately 30–60 min).
3. Take a 1 ml sample immediately before induction (noninduced control), pellet cells, and resuspend in 50 µl 1x SDS-PAGE sample buffer. Freeze the sample at –20°C until needed for SDS-PAGE.
4. Induce expression by adding IPTG to a final concentration of 1 mM.
5. Grow the cultures for an additional 4–5 hours. Collect a second 1 ml sample (induced control), pellet cells and resuspend in 100 µl 1x SDS-PAGE sample buffer. Freeze until use.
6. Harvest the cells by centrifugation at 4000 x g for 20 min.

Protein extraction
1. Resuspend cell pellet in 5 ml of lysis buffer for native purification.
2. Freeze sample in dry ice/ethanol, and thaw in cold water.
   Alternatively, add lysozyme to 1 mg/ml and incubate on ice for 30 min.
3. Sonicate 6 x 10 s with 10 s pauses at 200–300 W. Keep lysate on ice at all times.
   Use a sonicator with a microtip probe.
4. Centrifuge lysate at 10,000 x g at 4°C for 20–30 min. Decant the supernatant (crude extract A, soluble protein) and save on ice.
5. Resuspend the pellet in 5 ml lysis buffer. This is a suspension of the insoluble matter (crude extract B, insoluble protein).
SDS-PAGE analysis

1. Add 5 µl of 2x SDS-PAGE sample buffer to 5 µl of crude extracts A & B.
2. Heat these samples, along with the frozen noninduced and induced cell samples at 95°C for 5 min.
3. Microcentrifuge at 15,000 x g for 1 min.
4. Load 20 µl of the noninduced and induced cell samples, and all of the extract samples on a 12% SDS-PAGE gel. Run the gel according to standard procedures.

Interpretation of results

If the protein of interest is in the insoluble matter (extract B), ensure that the cells are completely lysed. If the protein is still insoluble, try extracting the pellet with 0.25% Tween 20, 0.1 mM EGTA a few times; often the protein is not truly insoluble but just associated with the membrane fragments in the cell pellet. If the protein is truly insoluble under these conditions, purify under denaturing conditions.
Protocol 7. Growth of standard *E.coli* expression cultures (100 ml)

1. Inoculate 10 ml of culture medium containing both ampicillin (100 µg/ml) and kanamycin (25 µg/ml) in a 50 ml flask. Grow the cultures at 37°C overnight. Kanamycin should be omitted when using the cis-repressed pQE-80L series of vectors.

2. Inoculate 100 ml of prewarmed media (with antibiotics) with 5 ml of the overnight cultures and grow at 37°C with vigorous shaking until an OD$_{600}$ of 0.6 is reached (30–60 min).

3. Take a 1 ml sample immediately before induction. This sample is the noninduced control; pellet cells and resuspend them in 50 µl 5x SDS-PAGE sample buffer. Freeze until SDS-PAGE analysis.

4. Induce expression by adding IPTG to a final concentration of 1 mM.

5. Incubate the cultures for an additional 4–5 h. Collect a second 1 ml sample. This sample is the induced control; pellet cells and resuspend in 100 µl 5x SDS-PAGE sample buffer. Freeze and store the sample at −20°C until SDS-PAGE analysis.

6. Harvest the cells by centrifugation at 4000 x g for 20 min.

7. Freeze the cells in dry ice–ethanol or liquid nitrogen, or store cell pellet overnight at −20°C.

Protocol 8. *E.coli* culture growth for preparative purification (1 liter)

1. Inoculate 20 ml of LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Grow at 37°C overnight with vigorous shaking. Kanamycin should be omitted when using the cis-repressed pQE-80L series of vectors.

2. Inoculate a 1 liter culture (LB, 100 µg/ml ampicillin, 25 µg/ml kanamycin) 1:50 with the noninduced overnight culture. Grow at 37°C with vigorous shaking until an OD$_{600}$ of 0.6 is reached.

3. Take a 1 ml sample immediately before induction. This sample is the noninduced control; pellet cells and resuspend in 50 µl 5x SDS-PAGE sample buffer. Freeze until use.

4. Induce expression by adding IPTG to a final concentration of 1 mM.

5. Incubate the culture for an additional 4–5 h. Collect a second 1 ml sample. This is the induced control; pellet cells in a microcentrifuge and resuspend in 100 µl 5x PAGE sample buffer. Freeze until use.

6. Harvest the cells by centrifugation at 4000 x g for 20 min.

7. Freeze the cells in dry ice–ethanol or liquid nitrogen, or store cell pellet overnight at −20°C.
### Troubleshooting: expression

#### Comments and suggestions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Suggestion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No or low expression</strong></td>
<td></td>
</tr>
<tr>
<td>Protein is poorly expressed.</td>
<td>Check that the protein is not found in the insoluble fraction. Review “Specific considerations” beginning on page 51.</td>
</tr>
<tr>
<td>Culture conditions for expression are incorrect.</td>
<td>Use the same culture conditions and host cells to check the expression of DHFR encoded by a control plasmid (pQE-40).</td>
</tr>
<tr>
<td>Coding sequence is ligated into the incorrect reading frame.</td>
<td>Sequence the ligated junctions.</td>
</tr>
<tr>
<td>Protein is secreted.</td>
<td></td>
</tr>
<tr>
<td>Protein is rapidly degraded.</td>
<td></td>
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| **Inclusion bodies are formed** | |
| Expression level is too high (protein cannot fold correctly). | Reduce expression levels by modifying growth and induction conditions (see “High expression levels, insoluble proteins, and inclusion bodies” on page 53). |
| Protein is insoluble. | |
| Protein is highly toxic. | |

**Note:** It may not be necessary to use denaturing conditions for purification if the protein of interest is insoluble or has formed inclusion bodies. Check the levels of soluble protein remaining in the cytoplasm. This may be purified with Ni-NTA matrices.