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# I. About the System

## A. Description

The pET System is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after induction. Although this system is extremely powerful, it is also possible to attenuate the expression level simply by lowering the concentration of inducer. Decreasing the expression level may enhance the soluble yield of some target proteins. Another important benefit of this system is its ability to maintain target genes transcriptionally silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host, target protein expression may be initiated either by infecting the host with  $\lambda$ CE6, a phage that carries the T7 RNA polymerase gene under the control of the  $\lambda p_L$  and  $p_I$  promoters, or by transferring the plasmid into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control. In the second case, expression is induced by the addition of IPTG or lactose to the bacterial culture or using an autoinduction medium. Although in some cases (e.g., with innocuous target proteins) it may be possible to clone directly into expression hosts, this approach is not recommended as a general strategy. Two types of T7 promoters and several hosts that differ in their stringency of suppressing basal expression levels are available, providing great flexibility and the ability to optimize the expression of a wide variety of target genes.

All of the pET vectors and companion products are available as kits designed for convenient cloning, expression, detection, and purification of target proteins. The pET Expression Systems provide the plasmids and host strains. The background information in Section VII, *Additional Guidelines*, will help you determine the best vector/host combination for your application.

## B. Licensing and Use Agreement

This T7 expression system, including bacteria, phages, and plasmids that carry the gene for T7 RNA polymerase, is made available under the conditions listed in the Academic and Non-profit Laboratory Assurance Letter. Please refer to the complete list of conditions on page 71.

## C. System Components

pET Expression Systems provide the core reagents needed for target gene cloning and expression.

- pET vector DNA, 10 µg each of the indicated plasmids
- Host bacterial strains BL21, BL21(DE3), and BL21(DE3)pLysS, glycerol stocks<sup>1,2</sup>
- Induction control clone, glycerol stock

*A good way to distinguish glycerol stocks from competent cells: glycerol stocks are supplied in screw-top tubes; competent cells have a flip top.*

**Systems plus Competent Cells** include all of the above listed components and a set of three competent host strains ready for high-efficiency transformation of pET recombinants. The competent cells are sufficient for up to 10 transformations in each host:

- 0.2 ml each of NovaBlue, BL21(DE3), and BL21(DE3)pLysS Competent Cells<sup>1,2</sup>
- SOC medium
- Test Plasmid

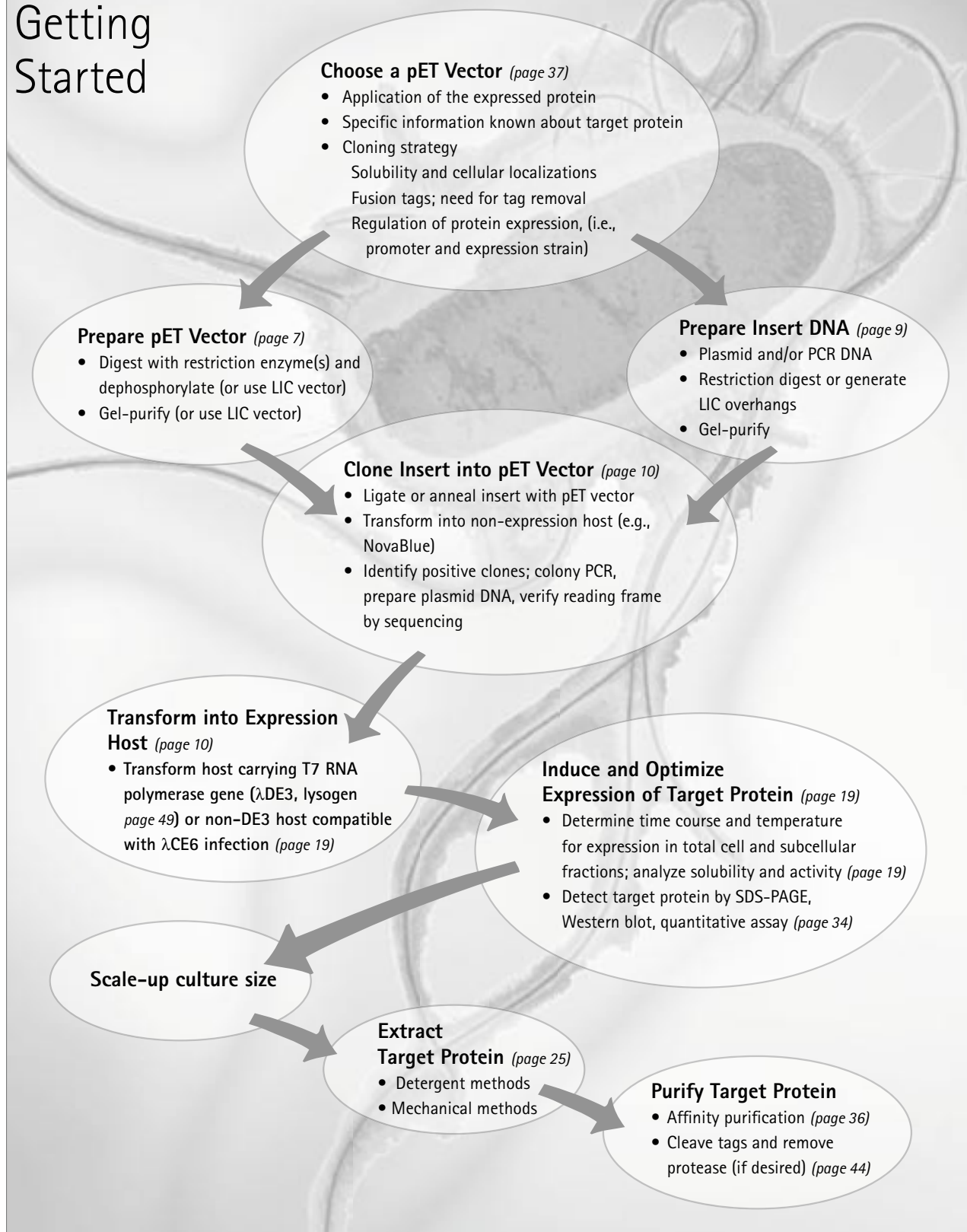
<sup>1</sup> The pET Peptide Expression System 31 includes host strains BLR and BLR(DE3)pLysS in place of the BL21 series hosts.

<sup>2</sup> The pET Trx Fusion System 32 includes the Origami™ series hosts strains in addition to the BL21 series hosts.

**Separate components and related products:** see the Novagen® catalog or Novagen website ([www.novagen.com](http://www.novagen.com)) for a complete listing of pET vectors, systems, and competent cells.

## II. Getting Started

### A. Overview of the pET System Process



## B. Growth Media and Antibiotics

### Growth media

A wide range of growth media is suitable for growth of strains and expression of target proteins in the pET System. The Overnight Express™ Autoinduction Systems enable regulated protein expression in *E. coli* without monitoring the culture or adding inducer during cell growth. These unique culture media are based on technology by F. William Studier at Brookhaven National Laboratory. The products include Overnight Express System 1 (Cat. No. 71300), System 2 (Cat. No. 71366), and Instant TB Medium (Cat. No. 71491). For more information see page 20.

Other suitable growth media include Luria-Bertani (LB) broth, Terrific Broth (TB), M9, and M9ZB. Recipes and stock solutions are shown below.

<b>LB</b>	
Per liter:	
10 g Tryptone	
5 g Yeast extract	
10 g NaCl	
• Adjust pH to 7.5 with 1N NaOH	
• Autoclave	
<b>TB (Sambrook et al., 1989)</b>	<b>K phosphate</b>
Per liter:	Per liter:
900 ml Deionized water	23.1 g $\text{KH}_2\text{PO}_4$
12 g Tryptone	125.4 g $\text{K}_2\text{HPO}_4$
24 g Yeast extract	• Autoclave
4 ml Glycerol	
• Autoclave, cool to 60°C	
• Add 100 ml sterile K phosphate	
<b>M9*</b>	<b>20X M9 salts</b>
Per liter:	Per liter:
50 ml 20X M9 salts	20 g $\text{NH}_4\text{Cl}$
20 ml 20% glucose	60 g $\text{KH}_2\text{PO}_4$
1 ml 1 M $\text{MgSO}_4$	120 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$
0.5 g NaCl	• Autoclave
930 ml autoclaved deionized $\text{H}_2\text{O}$	
<b>M9ZB (Studier et al., 1990)</b>	<b>10X M9 salts</b>
Per liter:	Per liter:
10 g N-Z-amine A (Quest)	10 g $\text{NH}_4\text{Cl}$
5 g NaCl	30 g $\text{KH}_2\text{PO}_4$
• Autoclave and cool	60 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$
• Add 100 ml 10X M9 salts, 1 ml 1M $\text{MgSO}_4$ , 10 ml 40% glucose (from autoclaved stocks)	• Autoclave

\* Add appropriate amino acids if desired host strain is an auxotroph.

**Antibiotics and stock solutions**

Stock solution	Preparation	Cat. No.
Ampicillin (sodium salt)	25 mg/ml in deionized water. Filter sterilize and store at -20°C. Use at 50 µg/ml within 1 month.	171254
Carbenicillin (disodium salt)	50 mg/ml in deionized water. Filter sterilize and store at -20°C. Use at 50 µg/ml.	69101-3
Chloramphenicol	34 mg/ml in ethanol. Store at -20°C. Use at 34 µg/ml.	220551
Kanamycin (sulfate)	30 mg/ml in deionized water. Filter sterilize and store at -20°C. Use at 30 µg/ml for cells containing kan <sup>R</sup> plasmids, and at 15 µg/ml for cells with a chromosomal kan <sup>R</sup> gene (i.e., Origami™ and Rosetta-gami™ strains).	420311
Rifampicin	10 mg/ml in 67% methanol, 0.17 N NaOH. Use at 200 µg/ml within 5 days. Protect from light.	557303
Spectinomycin, Dihydrochloride, Pentahydrate	50 mg/ml in deionized water. Filter sterilize and store at -20°C. Use at 50 µg/ml.	567570
Streptomycin Sulfate	50 mg/ml in deionized water. Filter sterilize and store at -20°C. Use at 50 µg/ml within 3 months.	5711
Tetracycline	5 mg/ml in ethanol. Store at -20°C. Use at 12.5 µg/ml.	58346
Glucose	20% (w/vol) D-glucose solution in H <sub>2</sub> O. Autoclave. Store sterile solution at room temperature. Add glucose to LB agar with antibiotics to a final concentration of 0.5–1% (see page 47).	346352
100 mM IPTG (isopropyl-D-thiogalactopyranoside)	2.38 g IPTG in 100 ml deionized water. Filter sterilize and store at -20°C.	70527-3*

\*100 mM IPTG Solution

**C. Vector Preparation**

For information on pET vector choices, please see "Choosing a pET Vector" starting on page 37.

For vector preparation, use the restriction enzyme manufacturer's recommended buffer and incubation conditions for the enzymes you are using. Many combinations of enzymes are compatible when used together in the same buffer.

- Note that different enzymes digest with different efficiencies, especially when two sites are close together. In general, enzymes with compatible buffers and whose sites are more than 10 bp apart can be used together in the same reaction. If one of the enzymes is a poor cutter, if the buffers are incompatible, or if the sites are separated by 10 bp or less, the digestions should be performed sequentially. The first digestion should be done with the enzyme that is the poorest cutter and the second enzyme added after digestion has been verified by running a sample of the reaction on an agarose gel.
- Note that some restriction enzymes may display "star activity," a less stringent sequence dependence that results in altered specificity. Conditions that can lead to star activity include high glycerol concentration (> 5%), high pH, and low ionic strength.

For more information on cloning strategies, see "pET Vector Cloning Strategies", starting on page 43.

As described in Section VII, Additional Guidelines, it is also possible to clone PCR products without restriction digestion using the ligation-independent cloning (LIC) method with Novagen Ek/LIC, Xa/LIC, and 3C/LIC Vector Kits. In this case, follow the protocols provided with the LIC Vector Kits.

- If cloning into a single site, dephosphorylate the vector following restriction

digestion to decrease the background of non-recombinants resulting from self-ligation of the vector. Molecular biology grade calf intestinal or shrimp alkaline phosphatase should be used according to the manufacturer's instructions.

- It is also useful to dephosphorylate vectors cut with two enzymes, especially when the sites are close together or if one of the enzymes is a poor cutter. This decreases the non-recombinant background caused by incomplete digestion with one of the enzymes, which is undetectable by gel analysis.
- Following digestion it is usually worthwhile to gel-purify the vector before insert ligation to remove residual nicked and supercoiled plasmid, which transform very efficiently relative to the desired ligation products. This step is optional, but usually reduces the effort required to screen for the correct construction. The SpinPrep™ Gel DNA Kit is ideal for rapid isolation of DNA fragments from agarose gel slices.

*To digest and gel-purify the vector:*

1. Assemble the following components in a microcentrifuge tube:

3 µg	pET vector
3 µl	10X restriction enzyme buffer
10–20 U	Each restriction enzyme (assuming compatible buffers; the total volume of enzyme added should not exceed 10% of the reaction volume to avoid high glycerol concentrations)
3 µl	1 mg/ml acetylated BSA (optional)
x µl	Nuclease-free water to volume
<hr/>	
30 µl	Total volume

2. Incubate at the appropriate temperature (usually 37°C) for 2–4 h.
3. Run a 3 µl sample together with Perfect DNA™ Markers on an agarose gel to evaluate the extent of digestion.
4. When digestion is complete, add calf intestinal alkaline phosphatase (Cat. No. 524576) directly to the remainder of the digestion. This enzyme functions in most restriction buffers under the conditions described here. It is important to use the correct amount of enzyme; too much can cause unwanted deletions and can be difficult to remove for future steps. 3 µg of a typical pET vector (5 kbp) corresponds to about 2 pmol DNA ends when linearized, or about 4 pmol ends if two enzymes were used for digestion. We recommend using 0.05 unit of alkaline phosphatase per pmol ends. Dilute the enzyme in water or 50 mM Tris-HCl, pH 9.0 just before use.
5. Incubate at 37°C for 30 min.
6. Add gel sample buffer to the reaction and load the entire sample into a large well (0.5–1.0 cm wide) on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Run the gel far enough to separate the linear plasmid from nicked and supercoiled species. It is useful to run uncut vector DNA in an adjacent lane to help distinguish undigested from linearized plasmid DNA.
7. Visualize the DNA band with a long wave UV light source and excise the band from the gel using a clean razor blade. Minimize exposure to the light source, which can cause nicks and double strand breaks in the DNA.

8. Recover the DNA from the gel slice. The SpinPrep™ Gel DNA Kit (Cat. No. 70852-3) is ideal for this application. Resuspend the final product in a total volume of 30  $\mu$ l (usually about 50 ng/ $\mu$ l DNA). The DNA can be quantified spectrophotometrically or using the PicoGreen® kit from Molecular Probes. Assume recoveries in the range of 50%.
9. Store the treated vector at  $-20^{\circ}\text{C}$  until use.

*Note: If the vector is not gel-purified or if the gel recovery method does not remove residual alkaline phosphatase, extract the reaction successively with 1 volume TE-buffered phenol, 1 volume TE-buffered phenol:CIAA (1:1; CIAA is chloroform:isoamyl alcohol, 24:1), and 1 volume CIAA. Then precipitate with 0.1 volume 3 M Na acetate and 2 volumes of ethanol. Centrifuge at 12,000  $\times$  g for 10 min, rinse the pellet with 70% ethanol, air dry, and resuspend in 30  $\mu$ l TE buffer. Pellet Paint® Co-Precipitant (Cat No. 69049-3) can be added to the precipitation for easy visualization of the pellet.*

## D. Insert Preparation

Preparing inserts by restriction digestion followed by gel purification is usually straightforward. Note that when subcloning into the pET vectors from vectors with the same selective marker (even with PCR as discussed below), it is necessary to gel-purify the fragment of interest to remove the original plasmid, which will transform very efficiently. As little as 10 pg of contaminating supercoiled plasmid (i.e., less DNA than can be visualized on an agarose gel) can typically result in many more colonies containing the original plasmid than the desired pET subclone.

PCR can be used to isolate and/or modify target genes for expression in pET plasmids. With this approach, it is possible to design primers that will (1) isolate the translated portion of a cDNA sequence, (2) add convenient restriction enzyme sites or LIC overhangs, and (3) place the coding region in the proper reading frame. In general, primers should contain a minimum of 15 (preferably 18–21) nucleotides complementary to the sequence of interest with a GC content of approximately 50%, and restriction sites should be flanked by 3–10 “spacer” nucleotides (depending on the enzyme) at the 5' end to allow for efficient digestion.

One risk in using PCR for insert preparation is the potential to introduce mutations. The error rate of the PCR can be minimized in several ways:

- Use an enzyme with high fidelity, such as KOD HiFi, Hot Start, or XL DNA Polymerases (Cat Nos. 71085, 71086, or 71087)
- Limit the number of PCR cycles.
- Increase the concentration of target DNA.
- Increase the primer concentration.

### III.

## Cloning Inserts in pET Vectors

Procedures and recommendations in this section discuss cloning an insert into a pET vector. This process includes ligation and transformation into a non-expression host, and analyzing your construct. The Clonables™ Ligation/Transformation Kit (Cat. No. 70526-3) contains pretested ligation mix and high efficiency competent cells. This kit is designed for convenient, reproducible ligation and transformation of a vector and insert with any type of ends (see User Protocol TB233). After the construct is verified, plasmid is transformed into an expression host for protein production.

### A. Ligation

- For a standard reaction using DNA fragments with 2–4 base sticky ends, use 50–100 ng (0.015–0.03 pmol) of pET vector with 0.2 pmol insert (e.g., 50 ng of a 500 bp fragment) in a volume of 20 µl. Assemble the following components in a 1.5-ml tube [available in the DNA Ligation Kit (Cat. No. 69838-3) or use the Clonables™ 2X Ligation Premix (Cat. No. 70573-3)]. Add the ligase last.
 

2 µl	10X Ligase Buffer (200 mM Tris-HCl, 100 mM MgCl <sub>2</sub> , 250 µg/ml acetylated BSA, pH 7.6)
2 µl	100 mM DTT
1 µl	10 mM ATP
2 µl	50 ng/µl prepared pET vector
x µl	Prepared target gene insert (0.2 pmol)
y µl	Nuclease-free water to volume
1 µl	T4 DNA Ligase, diluted (with Ligase Dilution Buffer) to 0.2–0.4 Weiss U/µl
<hr/>	
20 µl	Total volume
- Gently mix by stirring with a pipet tip. Incubate at 16°C for 2 h to overnight. Also set up a control reaction in which the insert is omitted to check for non-recombinant background.

*Note:* For blunt ends, use 10X more ligase (i.e., undiluted enzyme), reduce the ATP concentration to 0.1 mM, and incubate for 6–16 h at 16°C or 2 h at room temperature.

### B. Transformation

We recommend using NovaBlue as the initial cloning host for pET vectors. NovaBlue is a convenient host for initial cloning of target DNA due to its high transformation efficiency and the high yields of high quality DNA that results from *recA endA* mutations. NovaBlue contains no source of T7 RNA polymerase, making it ideal for the establishment of recombinant plasmids under nonexpression conditions.

Competent cells in standard kits are provided in 0.2-ml aliquots. The standard transformation reaction uses 20 µl, so each tube contains enough cells for 10 transformations. Singles™ competent cells are provided in 50-µl aliquots, which are used “as is” for single 50-µl transformations. Please note that there are a few steps in the

protocol that vary for the Singles™ vs. standard kits. Novagen® NovaBlue and BL21(DE3) Competent Cells are also offered in a high-throughput 96-well plate format known as HT96™ Competent Cells (see User Protocol TB313).

DNA in ligation reactions containing high-quality reagents is suitable for direct addition to Novagen® competent cells. Inactivation of the ligase is not required before transformation. For transformation, 1 µl of the ligation reaction usually yields sufficient numbers of colonies for screening. Up to 1 µl (standard competent cells) or 5 µl (Singles competent cells) of the ligation reaction containing high-quality reagents can be added to the transformation without reducing transformation efficiency.

Plasmid DNA isolated using standard miniprep procedures is also usually satisfactory; however, for maximum efficiency, the sample DNA should be free of phenol, ethanol, salts, protein, and detergents, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or in water. Transformation efficiencies will generally be 10- to 100-fold higher with supercoiled plasmids than with ligation reactions, so it is often necessary to dilute standard plasmid preparations in TE buffer or water prior to transformation. One microliter containing 1–10 ng plasmid DNA is usually sufficient to produce hundreds of colonies. For cotransformations into expression strains using two supercoiled plasmids, add 1 µl containing 10–40 ng of each plasmid into expression strain competent cells. Higher concentrations of DNA will yield a higher number of transformants on the plate, but the transformation efficiency of the cells will decrease.

### Handling tips

1. Upon receipt, verify that the competent cells are still frozen and that dry ice is still present in the shipping container. Immediately place the competent cells at  $-70^{\circ}\text{C}$  or below. For optimal results, do not allow the cells to thaw at any time prior to use.
2. Handle only the rim of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible.
3. To mix cells, finger-flick the tube 1–3 times. NEVER vortex the competent cells.
4. Cells can be refrozen at  $-70^{\circ}\text{C}$  and used at a later date; however, transformation efficiencies may decline several-fold with each freeze-thaw cycle. To avoid multiple freeze-thaw cycles of the standard cells, dispense the cells into aliquots after the initial thaw and store the aliquots at  $-70^{\circ}\text{C}$  or below. To dispense cells from the 0.2 ml stock, remove the stock tube from the ice and finger-flick 1–3 times to mix prior to opening the tube. Remove a 20-µl aliquot from the middle of the cells and replace the tube immediately on ice. Dispense the aliquot immediately into the bottom of a chilled 1.5-ml tube, then immediately close the tube and replace on ice. Repeat until the entire 0.2 ml stock is dispensed into 20 µl aliquots. After all the aliquots have been dispensed, return any tubes that will not be immediately used for transformation to the freezer before proceeding.

**Procedure**

Prepare LB agar plates with appropriate antibiotic(s) ahead of time.

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is immersed in ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells to see that they have thawed and gently finger-flick the tube 1–2 times to evenly resuspend the cells. The cells are then ready to remove an aliquot (Standard Kits), or for the addition of the DNA (Singles Kits).
- 3.

Standard Kits:

Place the required number of 1.5-ml snap-cap polypropylene tubes on ice to pre-chill. Pipet 20 µl cells into the pre-chilled tubes.

Singles™ Kits:

Proceed to Step 4 or 5, depending on whether a Test Plasmid sample is included as a positive control.

4. **Optional:** To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
5. Add 1 µl of a ligation reaction or purified plasmid DNA directly to the cells. Stir gently to mix and return the tube to the ice. Repeat for additional samples.

*Note:* Transformation efficiencies may be increased several fold by diluting the ligation reaction 5-fold with TE or water prior to adding the DNA to the cells, or by extracting the ligation reaction twice with 1:1 TE-buffered phenol:CIAA (24:1 chloroform:isoamyl alcohol), once with CIAA, precipitating in the presence of sodium acetate, and resuspending in TE or water before adding the DNA to the cells.

6. Incubate the tubes on ice for 5 min.
7. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.

*Note:* This “heat shock” step is most easily accomplished if the tubes are in a floating rack in which the lower half of the tubes are exposed. Place the rack in the water bath so that the lower half of the tubes are submerged for 30 s, and then replace the rack on ice.

8. Place the tubes on ice for 2 min.
- 9.

Standard Kits:

Add 80 µl room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.

Singles Kits:

Add 250 µl room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.

10. Selection for transformants is accomplished by plating on medium containing antibiotic(s) for the plasmid-encoded drug resistance(s). Additional host-specific antibiotics also may be appropriate to insure maintenance of the host encoded feature(s) (see chart on page 52).

**When using NovaBlue:** if selecting for ampicillin or chloramphenicol resistance, plate 5–50 µl cells directly on selective media. If selecting for kanamycin or

streptomycin/spectinomycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating on selective media.

**When using strains other than NovaBlue:** incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective media.

**Notes:** *The outgrowth incubation is conveniently performed in a shaking incubator using a test tube rack anchored to the shaking platform. Place each transformation tube in an empty 13 mm × 100 mm glass test tube in the rack. The snap-caps on the transformation tubes prevent them from falling to the bottom of the test tubes, and all transformation tubes remain vertical.*

*During the outgrowth (or earlier if omitting outgrowth), place the plates at 37°C. If the plates contain a lot of moisture, place them cover-side up and open the cover approximately 1/3 of the way to allow the plates to dry for 30–45 min. If the plates do not need drying, keep them closed and place them cover-side down in the 37°C incubator for approximately 20 min prior to plating.*

11. Refer to “Plating Technique” in the subsequent section for specific instructions. Spread 5–50 µl of each transformation on LB agar plates containing the appropriate antibiotic(s) for the plasmid and host strain (see page 52). When plating less than 25 µl, first pipet 40–60 µl SOC onto the plate and then pipet the cells into the SOC.

**Important:** *The appropriate amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. For recombinants in NovaBlue, expect 10<sup>5</sup>–10<sup>7</sup> transformants per microgram plasmid, depending on the particular insert and the ligation efficiency. Transformations with the pETcoco™ plasmid typically require a plating volume of 50 µl to obtain sufficient colonies because the pETcoco plasmids are large (> 12,000 bp).*

**When using the Test Plasmid,** *plate no more than 5 µl of the final NovaBlue transformation mix. For strains with an efficiency of 2 × 10<sup>6</sup> pfu/ug, plate 10 µl. Plate the transformation mix in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampicillin resistance gene, bla).*

12. Set the plates on the bench for several minutes to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

**Note:** *Strains having thioredoxin reductase (trxB) and glutathione reductase (gor) mutations (i.e., Origami™ and Rosetta-gami™ strains) may take 24 h or longer for efficient colony formation. To avoid satellite colony formation, use carbenicillin, instead of ampicillin, for the selection of pET vectors encoding ampicillin resistance.*

## Plating techniques

1. Remove the plates from the incubator. If plating less than 25 µl of the transformation, we recommend plating onto a pool of SOC, which facilitates even colony distribution on the plate surface. Using a sterile pipet tip, place 40–60 µl of SOC in the center of a plate for a plating cushion.
2. To remove the transformation sample, finger-flick the transformation tube 5–8 times, open the cap and immediately remove the sample volume from the middle of the transformation reaction.

- Transfer the sample to the plate by dispensing the sample volume into the SOC cushion. After the sample is expelled, use the same tip to pipet up the sample volume of SOC from the cushion edge and dispense that fluid back into the cushion. (This effectively rinses out your pipet tip.)

### ColiRollers™ Plating Beads

*ColiRollers™ Plating Beads (Cat. No. 71013) are treated glass beads that eliminate the use of the spreader and alcohol flame while evenly and consistently distributing cells without damage.*

To use ColiRollers, simply dispense 10–20 beads per plate. The beads can be dispensed before or after pipetting the transformation mix on the plate. Cover the plate with its lid and move the plate back and forth several times. The rolling action of the beads distributes the cells. Several plates can be stacked up and shaken at one time. After all plates have been spread, discard the ColiRollers by inverting the plate over a collection container. Incubate as in step 4 below.

#### Standard spreader

- Completely immerse the plating spreader (bent glass rod or equivalent) into ethanol and flame to sterilize. After the flame is extinguished, allow the spreader to cool approximately 10 s prior to placing the spreader on the plate. To further cool the spreader before spreading the cells, place the spreader on the LB agar at the outside of the plate (not touching the pool of cells).
- Slowly* rotate the plate while supporting the weight of the spreader.

*Important: Do not press down on the spreader – use just enough contact to spread the cells.*

- Spread until the sample is evenly distributed on the plate. If the plates are fairly dry, the sample and cushion will quickly absorb into the plate. If the plates are wet, spread until the sample is evenly distributed. Do not continue to spread until the sample and cushion have absorbed completely into the plate, as overspreading is lethal to the cells. Instead, after spreading briefly, set the plates upright at room temperature for approximately 15 min prior to placing them inverted in the 37°C incubator. This will allow excess moisture to absorb into the plates.
- Incubate all plates, cover-side down, in a 37°C incubator for 15–18 h. To obtain larger colonies, extend the incubation time slightly (1–2 h), but beware of the potential for development of satellite colonies with extended incubations (usually > 36 h at 37°C). Satellites are not commonly observed when using carbenicillin or kanamycin.

## C. Storage of Strains

Permanent stocks of hosts and pET recombinants are best maintained as glycerol stocks. Note that high glycerol concentrations (> 10%) may lead to plasmid instability.

*To prepare stock cultures of host strains and pET recombinants:*

- Inoculate a single colony into 50 ml medium containing appropriate antibiotic(s) in a 250 ml flask.
- Incubate with vigorous shaking at 37°C during the day until the OD<sub>600</sub> reaches 0.6–0.8.
- Remove 0.9 ml and transfer to a cryovial, add 0.1 ml 80% glycerol.
- Mix well and store at –70°C.

To inoculate a culture from the frozen stock:

1. Scrape or melt a few microliters from the surface (use a sterile pipet tip or plastic culture loop).
2. Streak on an agar plate or inoculate liquid medium containing appropriate antibiotic(s).
3. Return the remainder to the  $-70^{\circ}\text{C}$  freezer without thawing.

## D. Analysis of pET Recombinants

If the subcloning was successful, there are usually many more colonies produced from ligation in the presence of the insert than with the negative control. However, the cloning may be successful even if the number of colonies on the two plates is roughly equivalent. There are several methods for analysis of transformants, including colony PCR, plasmid preparation and restriction analysis, sequencing, and *in vitro* transcription and translation.

Before growing colonies for plasmid isolation, the presence of the appropriate insert and its orientation can be determined using direct colony PCR. This additional step may be particularly helpful if a “dirty” (many extraneous bands), unpurified PCR product was cloned. To determine insert orientation and size, 5 pmol (1  $\mu\text{l}$ ) of the 5' vector-specific PCR primer is used with 5 pmol of the 3' insert-specific primer. In a second reaction, the 5' insert-specific primer and 3' vector-specific primer is used. The T7 Promoter Primer (Cat. No. 69348-3) and T7 Terminator Primer (Cat. No. 69337-3) are suitable as 5' and 3' vector-specific primers, respectively, for many pET vectors. Consult the appropriate vector map for suggested primers. Alternatively, just the vector-specific primers can be used in one reaction if insert orientation information is not desired.

### Ligation screening

To verify ligation between the vector and insert, a ligation reaction can be analyzed directly by PCR using vector-specific primers or a combination of vector-specific and insert-specific primers. This approach will not verify individual clones, which can be verified by colony PCR (see page 16).

1. Assemble the following components for ligation PCR:

1 $\mu\text{l}$	Ligation reaction diluted 1:10 in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (0.25–0.5 ng vector)
5 $\mu\text{l}$	10X NovaTaq™ Buffer with $\text{MgCl}_2$
1 $\mu\text{l}$	Upstream primer (5 pmol)
1 $\mu\text{l}$	Downstream primer (5 pmol)
1 $\mu\text{l}$	10 mM dNTP Mix
0.25 $\mu\text{l}$	NovaTaq DNA Polymerase (1.25 U)
40.75 $\mu\text{l}$	Nuclease-free water to volume
<hr/>	
50 $\mu\text{l}$	Total volume

2. Add the enzyme or DNA last to start the reaction and mix gently. If necessary, add 2 drops of mineral oil from a 200  $\mu$ l pipet tip to prevent evaporation. Optimal results are usually obtained by heating the assembled reaction to 80°C prior to addition of the enzyme or DNA. Use the following general cycling conditions for 30 cycles:
  - 1 min at 94°C
  - 1 min at the proper annealing temperature (usually 55°C for vector primers)
  - 2 min at 72°C
  - 6 min final extension at 72°C
3. To remove the oil overlay and inactivate the polymerase, add 100  $\mu$ l of chloroform, mix 30 s, and centrifuge for 1 min. Remove the top aqueous phase (which may appear cloudy) containing the DNA.
4. Add 5  $\mu$ l 10X loading dye to the top aqueous phase.
5. Load 10–25  $\mu$ l per lane on a 1% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide. Include at least one lane of Perfect DNA™ Markers. A strong band should appear that has a size corresponding to total number of bases between and including the primers.

### Colony screening

Colonies can be screened for inserts, without plasmid preparation, by direct colony PCR using vector-specific primers. For most pET vectors, appropriate primers for screening by colony PCR are the T7 Promoter Primer and the T7 Terminator Primer. Exceptions are pET-43.1a-c(+), pET-44a-c(+), and pET-50b(+) in which the S•Tag™ 18mer Primer (Cat. No. 70828-3) is recommended rather than the T7 Promoter Primer. The annealing sites for these and other vector-specific primers are indicated on the respective vector maps.

1. Pick a colony from an agar plate using a 200  $\mu$ l pipet tip or sterile toothpick. Choose colonies that are at least 1 mm in diameter and try to get as many cells as possible. If a copy of the colony is desired, touch the pipet tip to a plate before transferring.
2. Transfer the bacteria to a 0.5-ml tube containing 50  $\mu$ l of sterile water. Vortex to disperse the cells.
3. Place the tube in boiling water or a heat block at 99°C for 5 min to lyse the cells and denature DNases.
4. Centrifuge at 12,000  $\times$  g for 1 min to remove cell debris.
5. Transfer 10  $\mu$ l of the supernatant to a fresh 0.5 ml tube for PCR. Leave on ice until use.

6. Prepare a master mix for colony PCR by assembling the following components. To account for pipetting losses, it is convenient to multiply the amounts by X.5, where X is the number of reactions.

Per reaction:

31.75  $\mu$ l Nuclease-free water

1  $\mu$ l dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)

1  $\mu$ l upstream primer, 5 pmol/ $\mu$ l

1  $\mu$ l downstream primer, 5 pmol/ $\mu$ l

5  $\mu$ l 10X NovaTaq™ Buffer with MgCl<sub>2</sub>,

0.25  $\mu$ l NovaTaq DNA Polymerase (1.25 U)

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40  $\mu$ l Total volume

*Note:* For greatest specificity, and yield of long complex targets for PCR use KOD Hot Start and KOD XL DNA Polymerases respectively, using the recommended buffers and cycling conditions (see User Protocols TB341, TB342). KOD polymerases are not available for sale in Japan through Novagen.

7. Add 40  $\mu$ l of the master mix to each 10  $\mu$ l sample, mix gently, add 2 drops of mineral oil, cap the tubes and put the samples in a thermal cycler.

*Note:* As an optional step, a hot start procedure can be used in which the cell lysate samples are prewarmed to 80°C before the addition of the master mix.

8. Process in the thermal cycler for 35 cycles, as follows:
  - 1 min at 94°C
  - 1 min at the proper annealing temperature (usually 55°C for vector primers)
  - 2 min at 72°C
  - 6 min final extension at 72°C
9. To remove the oil overlay and inactivate the polymerase, add 100  $\mu$ l of chloroform, mix 30 s and centrifuge for 1 min. Remove the top aqueous phase (which may appear cloudy) containing the DNA.
10. Add 5  $\mu$ l 10X loading dye to the top aqueous phase.
11. Load 10–25  $\mu$ l per lane on a 1% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide. Include at least one lane of Perfect DNA™ Markers. A strong band should appear that has a size corresponding to the total number of bases between and including the primers.

## Plasmid preparation procedure

Plasmid DNA from candidate recombinants should be verified for the presence of the correct insert and reading frame prior to transformation of an expression host. Several methods available for analysis of transformants include colony PCR, restriction analysis, sequencing, and *in vitro* transcription and translation.

When isolating pET plasmids with Mobius™ kits, use the low-copy number protocol provided. Plasmid DNA isolated with Mobius kits is essentially RNase-free. However, plasmid DNA isolated with SpinPrep™ Plasmid Kits or kits from other manufacturers may require an additional phenol: CIAA extraction and ethanol precipitation to eliminate RNases before *in vitro* transcription and translation. Add TE to 100 µl and extract twice with TE-buffered phenol:CIAA (1:1; CIAA is 24 parts chloroform, 1 part isoamyl alcohol) and once with CIAA. Transfer the final aqueous phase to a fresh tube and add 0.1 volume 3 M Na acetate and 2 volumes ethanol. Mix and incubate at –20°C for 30 min, centrifuge 5 min at 12,000 x g, and remove the supernatant. Rinse the pellet with 70% ethanol, centrifuge 5 min at 12,000 x g, and remove the supernatant. Dry and resuspend the DNA in 30 µl TE. If desired, 2 µl Pellet Paint® or Pellet Paint NF Co-Precipitant can be added along with the TE buffer prior to extraction to facilitate recovery of the DNA (the –20°C incubation can be eliminated if using Pellet Paint).

The table below provides information for selecting an appropriate plasmid preparation kit.

Plasmid Preparation Kit	Scale	DNA Yield	Cat. No.	Size
Mobius 1000 Plasmid Kit	100 ml (high-copy)	> 1 mg (high-copy)	70854-3	2 rxn*
	250 ml–1.5 L (low-copy)	200 µg–1 mg (low-copy)	70853-3	10 rxn*
			70853-4	25 rxn*
Mobius 500 pET Plasmid Kit	500 ml culture	500 µg (low-copy)	70969-3	10 rxn
Mobius 200 Plasmid Kit	35 ml culture (high-copy or low-copy)	> 200 µg (high-copy) > 30 µg (low-copy)	70970-3	25 rxn
SpinPrep Plasmid Kit	1–3 ml culture	5–10 µg (high-copy)	70957-3	20 rxn
		0.25–1 µg (low-copy)	70851-3	100 rxn

\*The kit sizes described are for the 100 ml (high-copy) or 250 ml (low-copy) preparations. Additional buffers are required for > 250 ml (low-copy) scale (User Protocol TB279).

## Sequencing

*If the sequencing template is precipitated, the addition of Pellet Paint or Pellet Paint NF Co-Precipitant helps visualize the pellet. Use Pellet Paint NF (Cat. No. 70748) with rhodamine-based labeling methods (e.g., PE Applied Biosystem automated sequencers) and Pellet Paint (Cat. No. 69049) with Cy5™-based automated sequencers.*

Detailed protocols for sequencing with double stranded and single stranded templates are available from manufacturers of sequencing kits. Primers for sequencing are indicated on the pET vector maps available at [www.novagen.com/vector](http://www.novagen.com/vector).

It is possible to prepare single stranded DNA template from PCR products with the Strandase™ Kit (Cat. No. 69202-3) or from pET plasmids that carry the phage f1 origin of replication by infection with single stranded helper phage. The f1 origin in pET vectors is oriented such that the single stranded DNA produced will anneal with the T7 terminator primer. The required helper phage (strain R408 or M13K07) and protocols for infection and DNA isolation are available from a number of commercial suppliers. The NovaBlue host strain carries an F' and is therefore suitable for helper phage infection.

## IV. Expressing the Target Gene

Target protein can be expressed from pET recombinants by induction of the T7 or T7lac promoter. Induction can be achieved in the indicated host strain as detailed below. The following sections give general information and protocols for protein expression; for information on optimizing protein expression see page 45.

### A. Bacteriophage CE6

Expression can be induced from a host strain without a source of T7 RNA polymerase by infection with Bacteriophage CE6. CE6 is a lambda recombinant that carries the cloned polymerase gene under control of the phage  $p_L$  and  $p_I$  promoters, the *cI857* thermolabile repressor, and the *Sam7* lysis mutations (Studier et al., 1986). When CE6 infects an appropriate host, the newly made T7 RNA polymerase transcribes target DNA so actively that normal phage development cannot proceed. Although this method is less convenient than induction of DE3 lysogens, it can be used if target gene products are too toxic to be maintained any other way. No T7 RNA polymerase is present in the cell before infection, so it should be possible to express any target DNA that can be cloned under control of a T7 promoter in this way. For more information see User Protocol TB007.

### B. Expression Host Transformation

For transformation into an expression host (i.e., a  $\lambda$ DE3 lysogen), obtain or prepare appropriate competent cells and use 1  $\mu$ l of a 50-fold dilution (approx. 1 ng) of plasmid in sterile water or TE buffer and follow the transformation procedure on page 12. Streak transformants for single colonies and prepare glycerol stocks as described on page 14.

### C. Induction of $\lambda$ DE3 Lysogens with IPTG

After a target plasmid is established in a  $\lambda$ DE3 lysogen, expression of the target DNA is induced by the addition of IPTG to a growing culture. For pET constructions carrying the “plain” T7 promoter, a final concentration of 0.4 mM IPTG is recommended for full induction, while 1 mM IPTG is recommended for full induction with vectors having the T7lac promoter. An example of an induction protocol is presented below.

Some DE3 host strains allow regulation of the expression level simply by varying the concentration of IPTG added. The Tuner™(DE3), Origami™ B(DE3), and Rosetta-gami™ B(DE3) strains contain the lacY1 mutation eliminating the active transport of lactose into cells via lac permease. Therefore, IPTG induction results in uniform, concentration-dependent entry into all cells in the population. When using these strains, a range of IPTG concentrations from 25  $\mu$ M to 1 mM should be tested, and the induced cultures examined for target protein activity and solubility to establish the optimal IPTG concentration.

#### Preparation for induction

Pick a single colony from a freshly streaked plate and inoculate 50 ml LB containing the appropriate antibiotic(s) for the plasmid and host strain in a 250 ml Erlenmeyer flask. For good aeration, add medium up to only 20% of the total flask volume.

For more information on expression host strains, see “Hosts for Expression”, page 49.

For more information on expression regulation, see “Regulating Protein Expression in the pET System”, page 45.

*Overnight cultures should be avoided to prevent elevated levels of basal expression and depletion of the antibiotic(s) from the media. However, if cultures are grown overnight, 0.5–1% glucose may be added to the media in order to reduce target protein expression prior to induction.*

Alternatively, inoculate a single colony or a few microliters from a glycerol stock into 2 ml LB medium containing the appropriate antibiotic(s) for the plasmid and host strain. Incubate with shaking at 37°C until the OD<sub>600</sub> reaches 0.6–1.0. Store the culture at 4°C overnight. The following morning, collect the cells by centrifugation (30 s in a microcentrifuge). Resuspend the cells in 2 ml fresh medium plus antibiotic(s) and use this to inoculate 50 ml medium.

### Sample induction protocol

Below is an induction protocol for a 100 ml culture. This scale is convenient for initial target protein verification (see following section) but may be adjusted as desired.

1. Prepare a starter culture of the pET recombinant in a λDE3 lysogen as follows: inoculate 3 ml of appropriate media (containing antibiotics) in a culture tube with a single colony from a plate or sterile loop of cells from a glycerol stock.
2. Incubate at 37°C with shaking at 250 rpm to an OD<sub>600</sub> of approximately 0.5. Add the entire 3 ml culture to 100 ml medium containing antibiotic(s).
3. Shake the culture at the desired temperature until the OD<sub>600</sub> is approximately 0.5–1.0 (e.g., 2–3 h in LB broth, 37°C). Monitor the OD<sub>600</sub> during growth by removing aliquots aseptically.
4. Just prior to induction, split the 100 ml culture into 2 × 50 ml cultures. Add IPTG to one of the 50 ml cultures and use the other culture as an uninduced control. For plasmids having the T7lac promoter, add IPTG to 1 mM (500 μl of sterile 100 mM IPTG) or for “plain” T7 promoter vectors, use 200 μl IPTG for a final concentration of 0.4 mM. Vary the IPTG concentrations with lacY mutant strains (Tuner™, Rosetta-gami™ B, and Origami™ B). Incubate both cultures with shaking at the desired temperature for the appropriate amount of time. Note that when directing fusion proteins to the periplasmic space, leakage of the protein to the medium might be enhanced by prolonged inductions (16 h or more).

## D. Autoinduction of λDE3 Lysogens

The Overnight Express™ Autoinduction Systems are designed for high-level protein expression with pET (Grabski et al., 2003) and other IPTG-inducible bacterial expression systems (Studier, 2005) without the need to monitor cell growth. Cell mass and target protein yield are often increased several-fold compared to conventional protocols using IPTG induction. The method is based on media components that are metabolized differentially to promote growth to high density and automatically induce protein expression from lac promoters. The Overnight Express Autoinduction Systems are extremely convenient for routine expression of proteins in either complex or defined media. The systems are ideal for high-throughput parallel analysis of protein expression, solubility, and purification from multiple expression clones.

Protocols are given below for the preparation and use of Overnight Express Instant TB Medium (Cat. No. 71491), a complete granulated culture medium. The granules ensure rapid and uniform dissolution in water, preventing clumping of the medium and generation of airborne powder.

There are two additional Overnight Express Autoinduction Systems available. The Overnight Express System 1 (Cat. No. 71300) contains three components: OnEx™ Solution 1 (induction solution); OnEx Solution 2 (buffering solution); and OnEx Solution 3 (magnesium solution). Addition of these components to traditional glucose-free complex media such as LB broth or TB results in maximum yields of target protein with the pET system (Grabski et al., 2003). The Overnight Express System 2 (Cat. No. 71366) contains OnEx Solutions 1–3 plus three additional components: OnEx Solution 4 (metals mixture), OnEx Solution 5 (amino acid mixture lacking methionine, cysteine, and tyrosine), and OnEx Solution 6 (methionine solution). Adding these six components to sterile water results in a defined medium capable of promoting high cell densities, enabling autoinduction of expression, producing maximum soluble proteins yields, and, if desired, efficient labeling of target proteins by the addition of selenomethionyl. For more information on the Overnight Express Autoinduction Systems see User Protocol TB383.

### Overnight Express™ Instant TB Medium

Absorption of water by Overnight Express Instant TB Medium leads to changes in pH and eventually to clump formation. If the pH changes after prolonged storage, the pH can be adjusted (see below). However, medium that has formed clumps may have undergone chemical changes and should be discarded. Therefore, we recommend preparing all of the contents of an EasyPak as soon as the package is opened. DO NOT rehydrate portions of an EasyPak. Bottles of the dry medium should be tightly closed after use to prevent water adsorption.

1. Pour the entire EasyPak (60 g) contents into a 2-L glass container or measure the appropriate amount of Overnight Express Instant TB Medium (60 g/L) and place in a vessel at least twice the final volume.
2. Add 1 L deionized water and 10 ml glycerol per 60 g Overnight Express Instant TB Medium.
3. Swirl gently until the medium is dissolved.
4. **Optional:** Divide the rehydrated Overnight Express Instant TB Medium into final culture volumes.

*Note:* Proper aeration is important for efficient growth and induction. For vessel size recommendations, see *Cell culture guidelines*, page 22.

5. Heat the medium in a microwave oven on high power setting until bubbles start to appear (usually 2–3 min per 500 ml medium, in a 1500 W microwave oven). Continue to microwave for 15–30 s after bubbles start to appear. DO NOT let the medium boil over.

*Note:* If the vessel containing the medium is too large for a microwave, the medium can be autoclaved.

6. Set the vessel on a bench top and cool the medium to room temperature.
7. Use immediately or store covered at 4°C until use.

*Note:* Rehydrated Overnight Express Instant TB Medium that has been prepared by heating in a microwave or autoclave can be stored up to one week at 4°C before use. Warm to culture temperature before inoculation.

- Add appropriate antibiotic(s) for the host strain and plasmid prior to inoculation.

#### pH adjustment

Overnight Express Instant TB Medium should be pH  $6.9 \pm 0.2$ . If the pH has changed after prolonged storage, adjust the pH using the following protocol.

- Remove a sample (i.e., 50 ml) of the reconstituted culture medium.
- Adjust the pH to 6.9 by adding 1 N or 0.1 N HCl or NaOH. Record the volume of HCl or NaOH used.
- Calculate the volume of HCl or NaOH to adjust the pH of the remaining prepared culture medium.
- Under sterile conditions, add the calculated volume of HCl or NaOH.

#### Cell culture guidelines

These conditions may require optimization depending on the expression system, target protein, host strain, growth medium, temperature, culture volume, and orbital-shaking incubator used. The following protocols are based on BL21(DE3) cell culture.

*Note: It is important to grow cells to stationary phase when using the Overnight Express™ Systems. See “Additional Guidelines” page 23 for more information.*

#### Tube or flask cultures

Inoculate Overnight Express System medium, plus appropriate antibiotic(s), with an isolated colony from agar plates grown overnight at 37°C, or with 0.001 volume of a glycerol stock. Inoculation of cultures greater than 30 ml should employ multi-staging by generating a 5% (vol/vol) inoculum. Start the culture by single colony inoculation of 2 ml Overnight Express System medium, incubate with shaking 300 rpm to an OD<sub>600</sub> of approximately 0.5, and stage this 5% inoculum procedure to obtain the desired volume. Several stages and flasks of increasing size filled with medium (10-20% of the flask volume) may be required. The staging procedure will minimize the shock induced lag phase in growth caused by transfer of a small inoculum to a larger volume of fresh medium and diffusion of vitamins, minerals and cofactors from the cells (Hunt and Stieber).

The following culture volumes and vessels are recommended to achieve appropriate aeration.

Culture volume	Vessel
0.5 ml	12 mm × 75 mm sterile snap-cap tube (VWR International, Cat. No. 60819-728)
2 ml	17 mm × 100 mm sterile snap-cap tube (VWR International, Cat. No. 60819-761)
10 ml	125-ml Erlenmeyer flask
30 ml	250-ml Erlenmeyer flask
100 ml	500-ml baffled flask
200 ml	1-L baffled flask
500 ml	2.8-L baffled flask

### 96-well or 24-well plate cultures

Inoculate Overnight Express System™ medium plus appropriate antibiotic(s) with 0.001 volume of a glycerol stock or with an isolated colony (1 colony/well) from plates grown overnight at 37°C. Cover 96-well plates with an air-permeable sealer and incubate at 37°C, shaking at 300 rpm for approximately 16 h. Cover 24-well plates with BugStopper™ Venting Capmats (VWR International, Cat. No. 14217-208) and incubate at 37°C, shaking at 200 rpm for approximately 16 h.

The following culture volumes and vessels are recommended to achieve appropriate aeration.

Culture volume	Vessel
1 ml	Sterile 96-Well Deep Well Cultures Plates with Sealers (Cat. No. 71111-3)
5 ml	24-well culture plates (VWR International, Cat. No. 13503-190)

### Additional guidelines

**Glycerol stock preparation:** When growing cultures to prepare glycerol stocks that will be used to inoculate Overnight Express cultures, we recommend the addition of 0.5% glucose to a glucose-free medium (e.g., TB, LB broth, or 2X YT) to maintain plasmid stability. Grow the cells to an OD<sub>600</sub> of 0.6–0.8 and add 0.1 vol of sterile 80% glycerol. Mix well and store at –70°C.

**Aeration:** Efficient growth to saturation and utilization of carbon sources provided in Overnight Express medium requires vigorous agitation and proper aeration. Optimized culture volume:vessel dimension ratio is required for proper aeration.

**Temperature and length of incubation:** It is important to grow the cells to stationary phase when using the Overnight Express Systems. Using the cell culture guidelines above, stationary phase is usually reached as quickly as 8–10 hours, if the cultures are incubated at 37°C. When lower incubation temperatures are used, saturation may only be reached by incubation for 24 hours or more. Continued incubation for several hours after stationary phase appears to have no deleterious effects.

To export the target protein using the signal sequence leaders present in a number of pET vectors or to improve the yield of soluble protein, growth, and induction at 25°C or 30°C may be optimal.

**Bacterial strains:** Because lactose is used for induction, expression hosts should produce functional lac permease (encoded by the *lacY* gene) and β-galactosidase (encoded by the *lacZ* gene) for consistent results in both complex and defined media. *lacY* mutant strains will not efficiently transport lactose for induction and *lacZ* mutants will not convert a portion of the transported lactose into the allolactose inducer. Elevated levels of target gene expression in *lacY* and *lacZ* mutant strains may occur as cells approach stationary phase in some complex media, including Overnight Express medium. However, this induction may vary depending upon medium composition, cell growth stage, and nutrient availability, all of which affect pH and the levels of cyclic AMP and acetate (Grossman et al., 1998).

If using a plasmid with a T7lac promoter for expression, a host strain that does not contain a pLysS plasmid is recommended [e.g., BL21(DE3)]. The combination of the T7 lysozyme expressed by the pLysS plasmid and the lac repressor encoded by pET

vectors carrying the T7lac promoter results in significantly reduced levels of protein expression when using the Overnight Express™ Autoinduction Systems. When the “plain” T7 promoter is used, the low level of lysozyme provided by pLysS has little effect on expression of target proteins.

**Expression vectors:** Overnight Express Autoinduction Systems are compatible with pET bacterial expression vectors and other IPTG-inducible bacterial expression systems.

## V. Target Protein Verification

This section describes target protein isolation methods for analysis and purification. With each target protein, it is important to verify the production and localization and to estimate the yield in the culture medium or cell. A quick screen analysis of induced cultures with PopCulture® Reagent may be especially useful when screening a number of clones. The PopCulture quick screen allows for rapid characterization of target protein activity and analysis of expression levels directly in the culture medium, without cell harvest.

To facilitate verification, a small-scale analysis of total cell protein in the medium, periplasm, soluble cytoplasm, and insoluble cytoplasm is recommended. Results of this analysis may lead to further optimization of the induction conditions or large-scale induction and purification using one or several of the protein extraction techniques described in this section.

### Normalizing loading volumes for SDS-PAGE

To facilitate the gel and Western analysis, two worksheets are provided to record data and calculate normalized loading volumes for standard mini gels (page 35). This formula relies on accurate harvest  $OD_{600}$  readings and the sample concentration factor for each fraction. The sample concentration factor is the volume of original culture used to produce the fraction, divided by the final volume of the fraction.

### Optical density analysis of the induced culture

1. After induction and just before harvest, shake the culture well to ensure a homogeneous suspension and remove a 0.5–1 ml aliquot from the induced and uninduced cultures.
2. Determine the  $OD_{600}$  of the culture as accurately as possible. Dilute the aliquot in the same media used for growth so that the  $OD_{600}$  reading is between 0.1 and 0.8 (usually 1:5 to 1:10 dilution is sufficient). Zero the spectrophotometer with the same medium.
3. Record both the dilution factor and the  $OD_{600}$  reading on the attached worksheet (page 35).

## A. PopCulture® Quick Screen for Expression Level, Activity, and Solubility

Before harvesting the cells, target protein activity and expression levels can be quickly assessed directly, without centrifuging the cells, using PopCulture Reagent. In addition, the prepared extract can be centrifuged to analyze separate soluble and insoluble fractions. To perform the optional quick screen use a 1-ml sample of the culture and follow the PopCulture Reagent protocol on page 33. Alternatively, Novagen offers the RoboPop™ Solubility Screening Kit, designed for protein solubility screening of bacterial cultures in a 96-well format (Cat. No. 71255-3, see User Protocol TB362).

## B. Total Cell Protein (TCP) Fraction

The expression of target genes may be assessed quickly by analysis of total cell protein (TCP) on a SDS-polyacrylamide gel followed by Coomassie blue staining. A TCP sample should also be analyzed in parallel with the four fractions described below to serve as a control for recovery of the target protein.

1. Before harvesting the cells, take a 1 ml sample of well-mixed culture and centrifuge at  $10,000 \times g$  for 1 min. Remove and discard the supernatant. Let the pellet drain by inversion and tap the excess medium onto a paper towel.
2. Resuspend the pellet completely by mixing in 100  $\mu$ l of 1X phosphate-buffered saline (PBS), giving a concentration factor of 10X (1 ml of starting culture divided by 100  $\mu$ l final fraction volume).
3. Add 100  $\mu$ l of 4X SDS Sample Buffer (Cat. No. 70607-3) and sonicate with a microtip at the following settings: power level between 2–3, at 20–30% duty for 8–10 bursts (Branson Sonifier® 450; sonication conditions may vary with the equipment). Alternatively, pass the sample through a 27-gauge needle several times to reduce the viscosity.
4. Immediately heat the sample for 3 min at 85°C to denature the proteins and then store at –20°C until SDS-PAGE analysis.

## C. Medium Fraction

Analysis of the medium may be instructive when performing prolonged inductions, when expecting protein export, or target protein leakage from the cells is suspected. Many recombinant proteins that are directed to the periplasm often also end up in the medium through a poorly understood “leakage” phenomenon. In most cases, target protein in the medium is due to damage of the cell envelope rather than true secretion (Stader et al., 1990).

1. Add 40 ml of the culture to a pre-weighed tube and harvest the cells by centrifugation at  $10,000 \times g$  for 10 min at 4°C.
2. Carefully transfer 1 ml supernatant to a microcentrifuge tube. Avoid removing any cell pellet. The remaining medium can be saved for further assays. Place the cell pellet on ice until used to prepare the periplasmic fraction.
3. Concentrate the medium sample by either TCA precipitation or spin filter concentration, as follows:

Alternatively, the ProteoExtract™ Protein Precipitation Kit (Cat. No. 539180) quickly and efficiently concentrates and prepares protein from a variety of sources.

#### Trichloroacetic acid (TCA) precipitation

- a) Add 100 µl (1/10 volume) 100% TCA (w/v) to 1 ml medium and vortex for 15 s.
- b) Place on ice for a minimum of 15 min.
- c) Centrifuge at 14,000 × g for 10 min.
- d) Remove and discard the supernatant.
- e) Wash the pellet twice with 100 µl acetone, by adding the acetone, mixing, and then spinning for 5 min (14,000 × g). Remove and discard the acetone from the loose pellet. Allow the final pellet to air dry thoroughly (leave the tube open on the bench top or in a hood for about 60 min, or spin briefly in a Speed-Vac® evaporator [Thermo Savant]). The presence of residual acetone will make resuspension more difficult.
- f) Add 100 µl of 1X PBS (sample concentration factor = 10X) and 100 µl of 4X SDS Sample buffer (Cat. No. 70607-3). Resuspend by vigorous vortex mixing or sonication.
- g) Immediately heat for 3 min at 85°C to denature the proteins and then store at -20°C until SDS-PAGE analysis.

#### Spin filter concentration

- a) Use a low MW cut-off filter (10 kDa or lower) and follow the manufacturer's recommendations to concentrate 500 µl of medium to approximately 50 µl, yielding a concentration factor of approximately 10X.
- b) After centrifugation, determine the volume of the concentrated sample remaining, record the concentration factor on the worksheet provided and transfer to a clean tube.
- c) Rinse the spin filter membrane with hot (> 90°C) 2X SDS Sample Buffer using an amount equal in volume to the concentrated sample just removed from the spin filter. Pool the 2X SDS Sample Buffer membrane rinse with the concentrated sample.
- d) Immediately heat for 3 min at 85°C to denature the proteins and then store at -20°C until SDS-PAGE analysis.

*Note:* Leakage can be distinguished from cell lysis by assaying for the cytoplasmic enzyme glucose 6-phosphate dehydrogenase (Buttistuzzi et al., 1977). The level of this enzyme in the medium fraction is expected to be very low unless substantial cell lysis has occurred.

## D. Periplasmic Fraction

When using vectors with pelB or DsbA/C signal sequences, target proteins may be directed to the periplasmic space. The leader is necessary, but not sufficient for export into the periplasm. Cleavable signal sequences that mediate the export of *E. coli* proteins to the periplasm can be classified as either Signal Recognition Particle (SRP) dependent or non-SRP dependent. Signal sequences like the one found in DsbA tend to have high hydrophobicity and are involved in a rapid, cotranslational pathway. Non-SRP dependent signal sequences like those found in the PhoA and MalE promote export by a post-translational mechanism (Huber, et al., 2005). However, it is clear that translocation also

can depend on the mature domain of the target protein, which is recognized by SecB, the major chaperone of export. The following osmotic shock protocol (Ausubel et al., 1989) is a simple method of preparing the periplasmic fraction from  $\lambda$ DE3 lysogens. However, osmotic shock is not appropriate for use with host strains containing pLysS or pLysE because T7 lysozyme damages the cell wall, resulting in disruption of the inner membrane and release of the cytoplasmic fraction.

1. Resuspend the cell pellet (generated in step 2 of *Medium Fraction*, previous section) thoroughly in 30 ml of 30 mM Tris-HCl, 20% sucrose, pH 8. Then add 60  $\mu$ l 0.5 M EDTA, pH 8 (final concentration of 1 mM). Add a magnetic stir bar and stir slowly at room temperature for 10 min.
2. Collect the cells by centrifugation at 4°C for 10 min at 10,000  $\times$  g. Remove all of the supernatant and discard.
3. Thoroughly resuspend the pellet in 30 ml of ice-cold 5 mM MgSO<sub>4</sub> and stir the cell suspension slowly for 10 min on ice. During this step, the periplasmic proteins are released into the buffer.
4. Centrifuge at 4°C for 10 min at 10,000  $\times$  g to pellet the shocked cells. Transfer a 1-ml sample from the supernatant (periplasmic fraction) to a weighed microcentrifuge tube. Avoid removing any loose pellet with the supernatant.
5. The excess supernatant (periplasmic fraction) may be removed and saved for activity assays, if desired. Save the cell pellet on ice for further processing of the soluble and insoluble cytoplasmic fractions in the following sections. Record the weight of the pellet.
6. Concentrate the periplasmic fraction by TCA precipitation or spin filtration as described in step 3 in the previous section (*Medium Fraction*). Again the desired concentration factor is 10X.
7. Add an equal volume of 4X SDS Sample Buffer (Cat. No. 70607-3) and immediately heat for 3 min at 85°C to denature the proteins. Store at -20°C until SDS-PAGE analysis.

**Note:** *The success of this procedure can be monitored with a light microscope by comparing the shape of the cells before and after the osmotic shock. Prior to the shock, the cells should be rod shaped. After the procedure the cells should be round/spherical. Also, osmotic shock-mediated release of periplasmic proteins can be distinguished from general cell lysis by assaying for the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (Buttistuzzi et al., 1977). The level of this enzyme in the periplasmic fraction is expected to be very low unless substantial cell lysis has occurred.*

A slightly different procedure has been reported (LaVallie et al., 1993) for extraction of *trxA* fusion proteins, which can be expressed from pET-32 and pET-48b(+) vectors.

*To extract *trxA* fusion proteins:*

1. Resuspend induced cells in ice-cold 20 mM Tris-HCl, 2.5 mM EDTA, 20% sucrose, pH 8.0, to a concentration of 5 OD<sub>550</sub> U/ml and incubate on ice for 10 min.
2. Centrifuge for 30 s at 15,000  $\times$  g, decant the supernatant, and resuspend the pellet in the same volume of ice-cold 20 mM Tris-HCl, 2.5 mM EDTA, pH 8.0. Incubate on ice for 10 min. Note that EDTA is not compatible with His•Bind® Resin.
3. Centrifuge for 10 min at 15,000  $\times$  g. The supernatant is the periplasmic fraction. Analyze the supernatant and pellet by SDS-PAGE.

## E. Soluble Cytoplasmic Fraction

This section describes methods for isolating the soluble cytoplasmic fraction. The use of BugBuster® Protein Extraction Reagent is quick and may retain greater target protein activity than mechanical methods, which expose target proteins to heat and oxidation.

### BugBuster Master Mix treatment

BugBuster Protein Extraction Reagent is formulated for the gentle disruption of the cell wall of *E. coli* to release active proteins. It provides a simple, rapid, low-cost alternative to mechanical disruption methods such as French Press or sonication for releasing expressed target proteins for purification or other applications. The proprietary formulation utilizes a detergent mix that is capable of cell wall perforation without denaturing proteins.

There are several BugBuster formulations available (see Section XIV, *Appendix-Related Products* page 78). The following is a protocol for extraction using BugBuster Master Mix.

BugBuster Master Mix is an all-in-one reagent that combines BugBuster Protein Extraction Reagent with Benzonase® Nuclease and rLysozyme™ Solution. BugBuster Master Mix allows maximum recovery of active soluble proteins, with no need for dilution of the mix or addition of other reagents.

1. If medium and periplasmic fractions are not needed, harvest cells from liquid culture by centrifugation at  $10,000 \times g$  for 10 min using a weighed centrifuge tube. For small scale extractions (1.5 ml or less), centrifugation can be performed in a 1.5-ml tube at  $14,000$ – $16,000 \times g$ . Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.
2. Resuspend the pellet, from step 1 above or step 5 of “*Periplasmic Fraction*”, in BugBuster Master Mix by pipetting or gentle vortexing, using 5 ml reagent per gram of wet cell paste. This typically corresponds to about 2.5 ml per 50-ml culture. For small cultures use up to 1/5 culture volume for resuspension (e.g., use 300  $\mu$ l BugBuster Master Mix for 1.5-ml cultures). Using higher ratios of BugBuster Master Mix causes no adverse effects.
3. **Optional:** Add protease inhibitors. Protease inhibitors are compatible with BugBuster Master Mix. Serine protease inhibitors should be avoided if the target protein is to be treated with Thrombin, Factor Xa, or Recombinant Enterokinase. Cysteine protease inhibitors should be avoided if the target protein is to be treated with HRV 3C. Although purification may remove active inhibitors, dialysis or gel filtration is recommended before cleavage.
4. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature.

*Note:* The extract should not be viscous after incubation.

5. Centrifuge at  $4^\circ\text{C}$  for 20 min at  $16,000 \times g$  to remove insoluble cell debris. The pellet may be used to isolate the “*Insoluble Cytoplasmic Fraction*” page 31.
6. Transfer the supernatant to a fresh tube for analysis. Mix a sample of the supernatant with an equal volume of 4X SDS Sample Buffer (Cat. No. 70607-3). The remainder of the supernatant may be used for additional analysis or purification.

7. Immediately heat the sample containing SDS for 3 min at 85°C to denature proteins and then store at -20°C until SDS-PAGE analysis.

### rLysozyme™ Solution and freeze/thaw treatment

This protocol isolates soluble protein using rLysozyme (Cat. No. 71110-3) and a freeze/thaw of the cell pellet. If the bacterial strain contains a plasmid encoding lysozyme (e.g., pLysS or pLysE), additional lysozyme treatment is not necessary.

1. If medium and periplasmic fractions are not needed, harvest cells from liquid culture by centrifugation at 10,000 × g for 10 min using a pre-weighed centrifuge tube. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.
2. Using the pellet from step 1 or from step 5 “*Periplasmic Fraction*”, freeze the pellet completely at -20°C or -70°C.
3. Completely thaw and resuspend the cell pellet by pipetting up and down or gentle vortexing in room temperature lysis buffer (50 mM Tris-HCl or NaH<sub>2</sub>PO<sub>4</sub>, 5% glycerol, 50 mM NaCl, pH 7–8) using 7 ml lysis buffer per gram of wet cell paste. Add protease inhibitors if desired.

*Note:* DO NOT add rLysozyme solution until a uniform cell suspension has been obtained. The freeze/thaw step ruptures the cell membrane allowing rLysozyme to access the cell wall. If rLysozyme is added prematurely, the immediate viscosity increase will make complete cell resuspension difficult and incomplete lysis may result.

4. Add approximately 7.5 KU of rLysozyme per 1 ml of lysis buffer (45–60 KU/gram cell paste).
5. **Optional:** Add 1 µl (25 U) of Benzonase® Nuclease per 1 ml of lysis buffer used for resuspension. Benzonase is not recommended for nuclease free preparations. Other methods of reducing viscosity include shearing or precipitating the nucleic acids (Burgess, 1991).
6. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature. Longer incubation time may be required if lysis is performed at 4°C. Determine empirically.

*Note:* If Benzonase® Nuclease was added, the extract should not be viscous at the end of incubation.

7. Remove insoluble cell debris by centrifugation at 4°C for 20 min at 16,000 × g. The pellet may be used to isolate the “*Insoluble Cytoplasmic Fraction*” page 32.
8. Transfer the supernatant to a clean tube for analysis and/or purification. Combine an equal volume of the supernatant with 4X SDS Sample Buffer (Cat. No. 70607-3) for SDS-PAGE Analysis. Additional analysis and/or purification can be performed with the remainder of the soluble extract. Maintain clarified extracts on ice for short-term storage (a few hours) or freeze at -20°C until needed.
9. Immediately heat the sample containing SDS for 3 min at 85°C to denature proteins and store at -20°C until SDS-PAGE analysis.

### Mechanical disruption

1. a) If medium and periplasmic fractions are not needed, harvest the cells from liquid medium by centrifugation for 10 min at 10,000 × g. Decant and allow the pellet

to drain, removing as much liquid as possible. Completely resuspend the pellet in 4-ml cold 20 mM Tris-HCl, pH 7.5 to give a concentration factor of 10X (40-ml culture to 4-ml buffer volume).

or

b) Completely resuspend the pellet from step 4 of the “*Periplasmic Fraction*” in 4 ml cold 20 mM Tris-HCl pH 7.5 to give a sample concentration factor of 10X (40-ml culture to 4 ml buffer volume).

*Note:* Some proteins may exhibit greater solubility when the cells are lysed in a buffer containing salt. Up to 0.5 M NaCl may be added to this buffer. Other proteins, such as those associated with membranes, may partition into the soluble fraction if a zwitterionic detergent (e.g., 10 mM CHAPS), is added to the lysis buffer.

## 2. Optional:

a) For each 1 ml of lysis buffer, add 3  $\mu$ l Lysonase™ Bioprocessing Reagent (Cat. No. 71230), an optimized ready to use mix of rLysozyme™ Solution and Benzonase® Nuclease. Incubate at 30°C for 15 min prior to sonication.

b) Add protease inhibitors. Protease inhibitors are compatible with Lysonase. Serine protease inhibitors should be avoided if the target protein is to be treated with Thrombin, Factor Xa, or Recombinant Enterokinase. Cysteine protease inhibitors should be avoided if the target protein is to be treated with HRV 3C. Although purification may remove active inhibitors, dialysis or gel filtration is recommended before cleavage.

## 3. Completely lyse the cells by one of the following methods:

a) *French Press*. Perform two passes at 20,000 psi using a chilled pressure cell.

b) *Sonication*. Mix the resuspended pellet by swirling and sonicate on ice using a microtip with the power level set between 4–5, at 40–50% duty for 15–20 bursts. It is important to keep the sample cold during sonication to avoid heat denaturation of proteins. The above settings are general recommendations and may need to be adjusted depending on the energy output of a given sonicator.

*Note:* Optimal cell disruption conditions for a sonicator may be quickly determined by performing a time course analysis. Remove samples at various times during the sonication, centrifuge for 5 min at 12,000  $\times$  g, and determine the protein concentration in the supernatant by a standard assay (e.g., Bradford, BCA, etc). When the protein concentration in the supernatant reaches a plateau, proceed to the next step.

4. Centrifuge the entire lysate or a 1.5 ml sample of the lysate (for normalized SDS-PAGE analysis) for 10 min at 14,000  $\times$  g to separate the soluble and insoluble fractions.

5. For normalized SDS-PAGE analysis, transfer 100  $\mu$ l of the soluble supernatant (from the 1.5 ml sample) to a new tube. Add 100  $\mu$ l of 4X SDS Sample Buffer (Cat. No. 70607-3). Immediately heat for 3 min at 85°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

6. Remove and save the remaining supernatant for activity assays or protein purification as desired.

7. Save the insoluble pellet fraction on ice for processing, as described in the next section.

## F. Insoluble Cytoplasmic Fraction

The insoluble cytoplasmic fraction may consist of cell debris and aggregated protein known as inclusion bodies. Inclusion bodies can be further purified by repeated centrifugation and washing steps. However, the product will be contaminated to some degree with other proteins and nucleic acids. In some cases, purified inclusion bodies can be used directly as antigens for the preparation of antibodies against the target protein (Harlow et al., 1988). Some target proteins associated with the insoluble fraction may not be in inclusion bodies. Membrane-associated target proteins can pellet with the insoluble fraction and may be released into the soluble fraction by including a detergent during lysis.

### Inclusion body purification after BugBuster® Master Mix treatment

1. Using the insoluble pellet from the “*BugBuster Master Mix treatment*” step 7, resuspend the pellet in the same volume of 1X BugBuster Master Mix that was used to resuspend the original cell pellet. Pipet up and down and vortex to obtain an even suspension. Complete resuspension of the pellet is critical to obtaining a high purity preparation in order to solubilize and remove contaminating proteins..
2. Add 6 volumes of 1:10 diluted BugBuster Master Mix (in deionized water) to the suspension and vortex for 1 min.
3. Centrifuge the suspension at 5,000 × g for 15 min at 4°C to collect the inclusion bodies. Remove the supernatant with a pipet.
4. Resuspend the inclusion bodies in half the original culture volume (0.5 vol) of 1:10 diluted BugBuster, mix by vortexing, and centrifuge as in step 3. Remove the supernatant. Repeat this step again. Resuspend once more but centrifuge at 4°C for 15 min at 16,000 × g, and remove the supernatant.
5. a) For normalized SDS-PAGE analysis, resuspend the final pellet in 1.5 ml 1% SDS with heating and vigorous mixing or sonication if necessary (resuspension in this volume maintains the concentration factor at 10X). Remove a 100-µl sample and combine with 100 µl 4X SDS Sample Buffer (Cat. No. 70607-3). Immediately heat for 3 min at 85°C to denature proteins and then store at -20°C until SDS-PAGE analysis.  
b) For purification, resuspend the pellet in the denaturing buffer of your choice, preferably using a buffer compatible with the desired purification method. The final pellet of inclusion bodies is compatible with resuspension in 1X Solubilization Buffer provided in the Protein Refolding Kit (see User Protocol TB234) or other denaturing buffers.

### Inclusion body purification after mechanical cell lysis or rLysozyme™ treatment

#### For normalized SDS-PAGE analysis

1. Wash the insoluble pellet by resuspending in 750 µl (from a 40-ml culture) 20 mM Tris-HCl, pH 7.5. Centrifuge at 10,000 × g for 5 min, remove the supernatant and repeat the wash step.
2. Resuspend the final pellet in 1.5 ml 1% SDS with heating and vigorous mixing or sonication if necessary (resuspension in this volume maintains the concentration factor at 10X).
3. Remove a 100 µl sample and combine with 100 µl 4X SDS Sample Buffer (Cat. No. 70607-3). Immediately heat for 3 min at 85°C to denature proteins and then store at -20°C until SDS-PAGE analysis.

#### For protein purification

1. Wash the insoluble pellet by resuspending in 16 ml purification buffer without denaturant (per 40 ml culture volume). Use a buffer that is appropriate for the purification resin.
2. Sonicate briefly to resuspend the pellet thoroughly and shear DNA.
3. Centrifuge at 5,000 × g for 15 min to collect the inclusion bodies and cellular debris while leaving other proteins in solution.
4. Remove the supernatant and resuspend the pellet in 20 ml buffer without denaturant (per 40 ml culture volume). Repeat Step 3. Sonication may be necessary to resuspend the pellet. Sometimes repeating this step several times releases more trapped proteins.
5. Remove the supernatant after the final centrifugation and resuspend the pellet in 5 ml buffer containing a denaturant to solubilize the inclusion bodies, preferably using a buffer compatible with the desired purification method. In some cases, incubating the sample on ice up to 1 h may help to solubilize the inclusion bodies. The final pellet is compatible with resuspension in 1X Solubilization Buffer provided in the Protein Refolding Kit (see User Protocol TB234) or other denaturing buffers.
6. Full or partially denatured inclusion bodies may be directly purified using His•Tag® fusion proteins (User Protocol TB054) or S•Tag™ fusion proteins (partially denatured, User Protocols TB160, TB087). Denatured proteins may be refolded prior to affinity purification (see User Protocol TB234).

## G. Preparation of Extracts with PopCulture® Reagent

PopCulture Reagent efficiently extracts protein from *E. coli* directly in the culture medium without cell harvest. Using this method, cell culture, protein extraction, and purification can all be performed in the original culture tube, flask, or multiwell plate (Grabski et al., 2001; Grabski et al., 2002).

An induced culture of *E. coli* is treated with PopCulture Reagent for 10–15 min at room temperature in the presence of rLysozyme™ Solution; additional treatment with

Benzonase® Nuclease is optional. The PopCulture extract can also be used directly in protein assays such as FRETWorks™ S•Tag™, S•Tag Rapid, or GST•Tag™ Assay Kits. This prepared extract can be directly combined with an equilibrated chromatography resins (e.g., GST•Mag™, GST•Bind™, Ni-NTA His•Bind®, His•Mag™) for purification. PopCulture® Reagent is also available in the RoboPop™ Purification Kits that include 96-well culture and collection plates for high throughput processing of cultures expressing His•Tag® or GST•Tag fusion proteins (see User Protocols TB327 and TB346).

1. Add 0.1 culture volume of PopCulture Reagent to an induced culture.
2. Add 40 U (1 µl of a 1:750 dilution) rLysozyme Solution per 1 ml culture volume to enhance cell lysis. Addition of rLysozyme is not necessary if the host strain contains pLysS or pLysE.

**Optional:**

Add 1 µl (25 U) Benzonase® Nuclease per 1 ml of original culture volume to degrade DNA and RNA for a non-viscous sample. rLysozyme and Benzonase can be pre-mixed with PopCulture prior to use.

3. Pipet up and down to mix and incubate for 10–15 min at room temperature.
4. This prepared extract can be assayed directly or combined with an equilibrated chromatography resin.

**SDS-PAGE of total cell protein:** Combine a sample of the prepared extract with an equal volume 4X SDS Sample Buffer (Cat. No. 70607-3) for detection with Coomassie staining or Western blotting. At an OD<sub>600</sub> between 3–5, a highly expressed protein may be detected with Coomassie staining using a 10 µl sample. Low expression levels and cell densities may require Western blotting.

**Quantitative assay:** Fusion tag specific quantitative assays such as FRETWorks™ S•Tag™ Assay, S•Tag Rapid Assay, and GST•Tag™ Assay are compatible with the PopCulture extracts, as are the BCA Protein Assay Kit and Bradford assays.

**Solubility:** To assess soluble and insoluble fractions, centrifuge the crude extract at 14,000 × g for 10 min to separate the fractions. *Soluble fraction:* A sample of the supernatant representing the soluble fraction can be assessed on by SDS-PAGE (as described above) or protein activity and quantitative assays may be performed. *Insoluble fraction:* Resuspend the pellet in 1 ml 1% SDS with heating and vigorous mixing or sonication. A sample of the solubilized pellet representing the insoluble fraction can be assessed by SDS-PAGE (as described above) or, if the target protein contains an S•Tag sequence, be assayed using the S•Tag Rapid Assay or FRETWorks S•Tag Assay.

**Target protein specific assays:** Because proteins generally retain their activities and conformation after PopCulture extraction, protein specific activity and immunoassays are likely to be possible.

**Purification:** PopCulture is Tris-buffered and is compatible with both Tris (His•Bind®) and phosphate (GST•Bind™, Ni-NTA His•Bind) buffer purification

systems at a neutral pH. The use of His•Mag™ or GST•Mag™ Agarose Beads enables the entire procedure (culture through purification) to be carried out in a single tube without columns or centrifugation. RoboPop™ Kits provide 96-well culture and purification plates, PopCulture® Reagent, and purification resins and buffers for His•Tag® or GST•Tag™ fusion proteins. (User Protocols TB327 and TB346). PopCulture is expected to be compatible with many other affinity purification resins.

## H. Detecting and Quantifying Target Proteins

*For a listing of detection and quantification products, see Section XIV, C, page 77.*

Protein expression can be determined by SDS-PAGE analysis of cell extracts followed by staining with Coomassie blue, which in many cases will reveal the target protein as a unique band when run adjacent to an uninduced extract. Western blotting is a more specific and sensitive method for identification of expression, and can be conveniently performed using fusion tag-specific reagents or protein-specific antibodies or other ligands. Activity assays are protein-dependent, especially in crude extracts, and are often performed following some degree of purification. However, crude extracts prepared with PopCulture® Reagent can be assayed immediately (see *Preparation of Extracts with PopCulture Reagent*, page 32).

### Normalized SDS-PAGE gel loading

Normalize the samples for loading based on the OD<sub>600</sub> at harvest so that a comparison of Coomassie-stained band intensity accurately reflects the relative amounts of target protein in various fractions. The Perfect Protein™ Markers (Cat. Nos. 69149-3 or 69079-3) or Trail Mix™ Markers (Cat. No. 70980-3) provide accurate size references for proteins between 15 kDa and 150 kDa (Cat. No. 69149-3) or 10 kDa and 225 kDa (Cat. Nos. 69079-3 and 70980-3) on gels stained with Coomassie blue.

*Note:* High levels of protein expressed in inclusion bodies can affect this normalization method. Cultures containing high levels of protein in inclusion bodies tend to give higher than expected OD readings. However, this method is useful for calculating the amount of sample needed to obtain similar protein loads for the different fractions.

*Worksheet 1:* Determination of the culture OD<sub>600</sub> at harvest.

	Dilution Factor (DF)	OD <sub>600</sub> of diluted sample	OD <sub>600</sub> at harvest (DF × OD <sub>600</sub> of diluted sample)
Induced Culture			
Uninduced Culture			

*Worksheet 2:* Determination of the normalized volume of sample to load on a 10-well or 15-well SDS-PAGE gel. The sample concentration factor represents the volume of original culture used to produce the fraction, divided by the final volume of the fraction. For example, if 1 ml of culture is used to prepare the fraction and after processing the final volume is 100 µl, then the sample concentration factor is 10. If larger gels are used, the loading volumes should be scaled up accordingly. The loading volume of each sample will need to be calculated, because the actual concentration factor for a given fraction may vary.

				Volume to Load	
				15-well mini-gel	10-well mini-gel
	Sample conc. factor	OD <sub>600</sub> at harvest	Z (conc. factor × OD <sub>600</sub> )	180 µl ÷ Z	270 µl ÷ Z
<b>Induced Samples</b>					
TCP					
Medium					
Periplasmic					
Soluble Cytoplasmic					
Insoluble					
<b>Uninduced Samples</b>					
TCP					
Periplasmic					
Medium					
Soluble Cytoplasmic					
Insoluble					

## VI. Purifying Target Proteins

*For a listing of affinity purification products see Section XIV, B, page 79.*

*For more information on vectors and host strains that can help increase solubility of target proteins, see Section VII, page 50.*

### A. Protein Purification

The methods chosen for protein purification depend on many variables, including the properties of the protein of interest, its location and form within the cell, the vector, host strain background, and the intended application for the expressed protein. Culture conditions can also have a dramatic effect on solubility and localization of a given target protein. Many approaches can be used to purify target proteins expressed with the pET System. One advantage of the system is that in many cases the target protein accumulates to such high levels that it constitutes a high percentage of the total cell protein. Therefore, it is relatively straightforward to isolate the protein in two or three chromatographic steps by conventional methods (ion exchange, gel filtration, etc.).

A variety of affinity purification methods are available that take advantage of the various peptide fusion tags available with pET vectors. In many cases, the use of an affinity method enables the purification of the target protein to near homogeneity in one step. Purification may include cleavage of part or all of the fusion tag with enterokinase, factor Xa, thrombin, or HRV 3C proteases. Before purification or activity measurements of an expressed target protein, preliminary analysis of expression levels, cellular localization, and solubility of the target protein should be performed using the methods described in Section V, “*Target Protein Verification*”, pages 24–35. The target protein may be found in any or all of the following fractions: soluble or insoluble cytoplasmic fractions, periplasm, or medium. Depending on the intended application, preferential localization to inclusion bodies, medium, or the periplasmic space can be advantageous for rapid purification by relatively simple procedures.

### B. Solubilization and Refolding Proteins

A variety of methods have been published describing refolding of insoluble proteins (Burgess, 1996; Frankel et al., 1996; Kurucz et al., 1995; Marston et al., 1990; Mukhopadhyay, 1997; Rudolph et al., 1996; Vincentelli et al., 2004; Willis et al., 2005). Most protocols describe the isolation of insoluble inclusion bodies by centrifugation followed by solubilization under denaturing conditions. The protein is then dialyzed or diluted into a non-denaturing buffer where refolding occurs. Because every protein possesses unique folding properties, the optimal refolding protocol for any given protein must be empirically determined. Optimal refolding conditions can be rapidly determined on a small scale by a matrix approach, in which variables, such as protein concentration, reducing agent, redox treatment, divalent cations, etc., are tested. Once the optimal concentrations are found, they can be applied to a larger scale solubilization and refolding of the target protein.

The Protein Refolding Kit uses a CAPS buffer at alkaline pH in combination with N-lauroylsarcosine to achieve solubility of the inclusion bodies, followed by dialysis in the presence of DTT to promote refolding. A discussion of various methods and factors involved in protein solubilization and refolding are included in User Protocol TB234, available at [www.novagen.com](http://www.novagen.com).

Depending on the target protein, expression conditions, and intended application, proteins solubilized from washed inclusion bodies may be > 90% homogeneous and may not require further purification. Purification under fully denaturing conditions (before refolding) is possible using His•Tag® fusion proteins and His•Bind® immobilized metal

affinity chromatography (see User Protocol TB054). In addition, S•Tag™, T7•Tag®, and Strep•Tag® II fusion proteins solubilized from inclusion bodies using 6 M urea can be purified under partially denaturing conditions by dilution to 2 M urea (S•Tag and T7•Tag) or 1 M urea (Strep•Tag II) prior to chromatography on the appropriate resin. Refolded fusion proteins can be affinity purified under native conditions using His•Tag, S•Tag, Strep•Tag II, and other appropriate affinity tags (e.g., GST•Tag™, and T7•Tag).

## VII. Additional Guidelines

### A. Choosing a pET Vector

The pET vectors were originally constructed by Studier and colleagues (Rosenberg et al., 1987; Studier et al., 1986; Studier et al., 1990). The latest pET vectors developed at Novagen offer enhanced features to permit easier cloning, detection, and purification of target proteins. There are two general categories of vectors available: transcription vectors and translation vectors.

- Transcription vectors are designed for expression of target genes that already carry their own prokaryotic ribosome binding site and AUG start codon. There are only three transcription vectors: pET-21(+), pET-23(+), and pET-24(+).
- Translation vectors contain the highly efficient ribosome binding site from the phage T7 major capsid protein and are used for the expression of target genes without their own ribosomal binding site. Review the *pET Vector Characteristic Table* for the entire selection of translation vectors (page 42).

The translation vector names are distinguished from the transcription vector names by the addition of a letter suffix following the name, e.g., pET-21a(+), which denotes the reading frame relative to the *Bam*H I cloning site recognition sequence, GGATCC. All vectors with the suffix “a” express from the GGA triplet, all vectors with the suffix “b” express from the GAT triplet, and all vectors with the suffix “c” express from the ATC triplet of the *Bam*H I recognition sequence. Vectors with a “d” suffix also express from the “c” frame, but contain an upstream *Nco* I cloning site in place of the *Nde* I site in that series for insertion of target genes directly into the AUG start codon.

#### Primary considerations

Choosing a pET vector for expression usually involves a combination of factors. Consider the following primary factors:

- The application intended for the expressed protein
- Specific information known about the expressed protein
- Cloning strategy

Applications for proteins expressed in pET vectors vary widely. For example, analytical amounts of a target protein may be needed for activity studies, screening and characterizing mutants, screening for ligand interactions, and antigen preparation. Large amounts of active protein may be required for structural studies, use as a reagent, or affinity matrix preparation. Any number of vectors may be suitable for expression of analytical amounts of protein for screening or antigen preparation, yet only one combination of vector, host strain, and culture conditions may work best for large-scale purification. If a high yield of active protein is needed on a continual basis, it is worth testing a matrix of vector, host, and culture conditions to find the optimal combination.

Any available information about the target protein may help determine the choice of vector. For example, some proteins require no extraneous sequence on one terminus or both termini for activity. Most pET vectors enable cloning of unfused sequences; however, expression levels may be affected if a particular translation initiation sequence is not efficiently utilized in *E. coli*. In these cases, an alternative is to construct a fusion protein with efficiently expressed amino-terminal sequences (indicated on the *pET Vector Characteristics Table*, page 42, with an N) and then remove the fusion tag by digestion with a site-specific protease following purification. Ligation-independent cloning (LIC) is especially useful for this strategy, because the cloning procedure enables removal of all or most amino-terminal vector-encoded sequences with either enterokinase, Factor Xa, or HRV 3C (as indicated in the *pET Vector Characteristics Table*, page 42).

Cloning strategies can affect the choice of vector due to the need for restriction site and reading frame compatibility. Because many of the pET vectors share common restriction site configurations, it is usually possible to clone a target gene into several vectors with a single preparation of the insert. Different considerations apply when using PCR cloning strategies. The LIC vector kits are recommended for this purpose, and enable the preparation of inserts by PCR and eliminate the need for restriction digestion of vector or insert.

### Solubility and cellular localization

Once the application and cloning strategy have been considered, a good starting point for any expression project is to determine the cellular localization and solubility of the target protein. In many applications, it is desirable to express proteins in their soluble, active form. Solubility of a particular target protein is determined by a variety of factors, including the individual protein sequence. In most cases, solubility is not an all-or-none phenomenon; the vector, host, and culture conditions can be used to increase or decrease the proportion of soluble and insoluble forms obtained.

The choice of vector and expression host can significantly increase the activity and amount of target protein present in the soluble fraction. A vector can enhance solubility and/or folding in one of three ways: 1) provide for fusion to a polypeptide that itself is highly soluble [e.g., glutathione-S-transferase (GST), thioredoxin (Trx), N utilization substance A (NusA)], 2) provide for fusion to an enzyme that catalyzes disulfide bond formation (e.g., thioredoxin, DsbA, DsbC), or 3) provide a signal sequence for translocation into the periplasmic space (pelB, DsbA, DsbC). When using vectors designed for cytoplasmic expression, folding can be improved in hosts that are permissive for the formation of disulfide bonds in the cytoplasm (e.g., *trxB* and *gor* mutations, see page 50).

An alternative strategy to obtain active, soluble proteins is to use vectors that enable export into the periplasm, which is a more favorable environment for folding and disulfide bond formation. For this purpose vectors carrying signal peptides are used. DsbA and DsbC are periplasmic enzymes that catalyze the formation and isomerization of disulfide bonds in pET-39b(+) and pET-40b(+), respectively. Other pET vectors that carry signal sequences without the additional DsbA or DsbC coding regions are also available (see chart on page 42).

In many cases target protein accumulates as insoluble inactive aggregates known as inclusion bodies. Inclusion bodies can be an advantage for purification because 1) they

are easily isolated by centrifugation to yield highly concentrated and relatively pure protein, and 2) inclusion body formation protects the protein from proteolytic attack. In addition, toxic proteins may not inhibit cell growth when present in inactive form as inclusion bodies.

Some purification strategies optimize production of insoluble inclusion bodies in the cytoplasm. Inclusion bodies are extracted and solubilized; then the target protein is refolded *in vitro*. This procedure usually produces the highest yields of initial protein mass and protects against proteolytic degradation in the host cell. However, the efficiency of refolding into active protein varies significantly with the individual protein and can be quite low. Therefore, this approach is often used for producing antigens or in other applications for which proper folding is not required. Also, pET-31b(+) is specifically designed for the generation of insoluble fusion proteins by fusion to Ketosteroid isomerase (KSI), a highly expressed hydrophobic domain. This provides a powerful method for the production of small proteins and peptides.

### Fusion tags

Fusion tags can facilitate detection and purification of the target protein, or may increase the probability of biological activity by affecting solubility in the cytoplasm or export to the periplasm. If a fusion sequence is tolerated by the application you are using, it is useful to produce fusion proteins carrying the GST•Tag™, His•Tag®, HSV•Tag®, Nus•Tag™, S•Tag™, Strep•Tag® II, T7•Tag®, or Trx•Tag™ peptides for easy detection on Western blots. Several of these peptides (fusion sequences) are small in size and the detection reagents for them are extremely specific and sensitive. The His•Tag, GST•Tag, S•Tag, Strep•Tag II, and T7•Tag sequences can also be used for affinity purification using the corresponding resin and buffer kits.

Fusion proteins can be accurately quantified in crude extracts or purified form using S•Tag and GST•Tag Assay Kits. The FRETWorks™ S•Tag Assay Kit is based on a novel substrate that enables fluorescent detection of less than 1 fmol of fusion protein in a homogenous format.

The His•Tag sequence is very useful as a fusion partner for protein purification. His•Tag fusion proteins can be affinity purified under fully denaturing conditions, which is particularly convenient for proteins expressed as inclusion bodies.

The Strep•Tag II sequence allows highly selective and easily controlled purification using Strep•Tactin®, a specifically engineered streptavidin. Gentle elution of purified recombinant protein is performed by addition of desthiobiotin (2.5 mM). Desthiobiotin is a reversibly binding and stable analog of biotin, the natural ligand of streptavidin.

The Nus•Tag, Trx•Tag, and GST•Tag sequences have been reported to enhance the solubility of their fusion partners. The Nus•Tag and Trx•Tag vectors together with *trxB/gor* mutant host strains (Origami™ strains and its derivatives), facilitate disulfide bond formation in the cytoplasm (see page 50), which may help maximize the level of soluble, active, properly folded target protein.

The various fusion tags and corresponding vectors are listed in the following table. A number of pET vectors carry several fusion tags in tandem as 5' fusion partners. In addition, many vectors enable expression of fusion proteins carrying a different

peptide tag on each end. Using vectors with protease cleavage sites (thrombin, Factor Xa, enterokinase, HRV 3C) between the 5' tag and the target sequence enables optional removal of one or more tags following purification. It should be noted that the expression of desired C-terminal fusions requires 1) the lack of a stop codon in the insert and 2) the proper reading frame at the cloning junction.

### Fusion Tags Available for pET Constructs

Tag	N/C Terminal or Internal (I)	Size (aa)	Basis for Detection and/or Purification	Applications	pET Vector Series
DsbA•Tag™ DsbC•Tag™	N	208 (DsbA) 236 (DsbC)	potential periplasmic localization	DB, DI, PE, SP	39, 40
GST•Tag™	N	220	monoclonal antibody, enzymatic activity, glutathione affinity	AP, IF, IP, QA, WB	41, 42, 49
His•Tag®	N, C, I	6, 8, or 10	monoclonal antibody, metal chelation chromatography (native or denaturing)	AP, IF, WB	14–16, 19–52
HSV•Tag®	C	11	monoclonal antibody	IF, WB	25, 27, 43.1, 44
KSI	N	125	highly expressed hydrophobic domain	PP	31
Nus•Tag™	N, I	495	promotes cytoplasmic solubility, monoclonal antibody	SP, WB	43.1, 44, 50
<i>pelB</i>	N	20	potential periplasmic localization	PE	20, 22, 25, 26, 27
PKA site	I	5	protein kinase A recognition site	PS	33
S•Tag™	N, I	15	S-protein (104 aa) affinity, monoclonal antibody	AP, IF, IP, QA, WB	29, 30, 32, 34–37, 39–50
Strep•Tag® II	N	8	monoclonal antibody, engineered streptavidin affinity	AP, WB	51, 52
T7•Tag®	N, I	11	monoclonal antibody	AP, IF, IP, WB	3, 9, 11, 17, 21, 23, 24, 28, 33
Trx•Tag™	N	109	monoclonal antibody, promotes disulfide bond formation	DB, SP	32, 48

AP: affinity purification  
DB: disulfide bond formation  
DI: disulfide bond isomerization  
WB: Western blotting

IP: immunoprecipitation  
PE: protein export  
PP: small protein/peptide production  
IF: immunofluorescence

QA: quantitative assay  
SP: soluble protein  
PS: *in vitro* phosphorylation

### Antibiotic resistance

The selective markers amp (ampicillin resistance, also abbreviated Ap or *bla* for  $\beta$ -lactamase gene) and kan (kanamycin resistance) are available with the pET vectors and are indicated in the table on page 42. Both types of selection have been widely used, but several simple guidelines are recommended when using vectors carrying the *bla* gene (see Section V, *Optimizing Expression*). While ampicillin resistance is commonly used for selection in a variety of cloning vectors, kanamycin resistance may be preferable under certain conditions, such as for protein expression in laboratories requiring GMP standards and when subcloning target genes from other ampicillin-resistant vectors. Ampicillin selection tends to be lost in cultures because secreted  $\beta$ -lactamase and the drop in pH that accompanies bacterial fermentation both degrade the drug. Some ways to avoid this loss of selection are to replace the medium with fresh ampicillin-containing medium or to use the related drug, carbenicillin, which is less sensitive to low pH.

Another difference between  $\text{kan}^{\text{R}}$  and most of the  $\text{amp}^{\text{R}}$  pET vectors involves the direction of transcription of the drug resistance gene. In  $\text{kan}^{\text{R}}$  pET vectors, the *kan* gene is in the opposite orientation of the T7 promoter, so induction of the T7 promoter should not result in an increase in *kan* gene product. In contrast, in some  $\text{amp}^{\text{R}}$  pET vectors the *bla* is located downstream and in the same orientation as the T7 promoter. All  $\text{amp}^{\text{R}}$  pET translation vectors have the native T7 transcription terminator (T $\phi$ ) located before *bla*. However, this terminator is only approximately 70% effective, allowing T7 RNA polymerase read-through to produce a small amount of  $\beta$ -lactamase RNA in addition to the target RNA. This results in the accumulation of  $\beta$ -lactamase enzyme in induced cultures. The orientation of *bla* has been reversed in the pET-43.1, pET-44, pET-45b, and pET-46 Ek/LIC, pET-51b, and pET-52b vectors, so that read-through by the T7 RNA polymerase will not result in increased levels of  $\beta$ -lactamase.

## pET Vector Characteristics Table

The following table lists the various cloning options available with the pET vectors. The (+) following the name indicates that the vector contains an f1 origin of replication that allows the production of single stranded plasmid DNA for mutagenesis and sequencing applications.

Vector	amp <sup>R</sup>	kan <sup>R</sup>	T7lac	His•Tag <sup>®</sup>	T7•Tag <sup>®</sup>	S•Tag <sup>™</sup>	Trx•Tag <sup>™</sup>	KSI	HSV•Tag <sup>®</sup>	PKA	Dsb•Tag <sup>™</sup>	GST•Tag <sup>™</sup>	Nus•Tag <sup>™</sup>	Strept•Tag <sup>®</sup> II	protease	signal seq.
pET-3a-d	●		●		N											
pET-9a-d		●	●		N											
pET-11a-d	●		●		N											
pET-14b	●		●	N											T	
pET-15b	●		●	N											T	
pET-16b	●		●	N											X	
pET-17b	●		●		N											
pET-19b	●		●	N											E	
pET-20b(+)	●		●	C												●
pET-21a-d(+)	●		●	C	N											
pET-22b(+)	●		●	C												●
pET-23a-d(+)	●		●	C	N											
pET-24a-d(+)		●	●	C	N											
pET-25b(+)	●		●	C					C							●
pET-26b(+)		●	●	C												●
pET-27b(+)		●	●	C					C							●
pET-28a-c(+)		●	●	N,C	I										T	
pET-29a-c(+)		●	●	C		N									T	
pET-30a-c(+)		●	●	N,C		I									T, E	
pET-30 Ek/LIC		●	●	N,C		I									T, E	
pET-30 Xa/LIC		●	●	N,C		I									T, X	
pET-31b(+)	●		●	C				N								
pET-32a-c(+)	●		●	I,C		I	N								T, E	
pET-32 Ek/LIC	●		●	I,C		I	N								T, E	
pET-32 Xa/LIC	●		●	I,C		I	N								T, X	
pET-33b(+)		●	●	N,C	I					I					T	
pET-39b(+)		●	●	I,C		I					N				T, E	●
pET-40b(+)		●	●	I,C		I					N				T, E	●
pET-41a-c(+)		●	●	I,C		I						N			T, E	
pET-41 Ek/LIC		●	●	I,C		I						N			T, E	
pET-42a-c(+)		●	●	I,C		I						N			T, X	
pET-43.1a-c(+)	●		●	I,C		I			C				N		T, E	
pET-43.1 Ek/LIC	●		●	I,C		I			C				N		T, E	
pET-44a-c(+)	●		●	N,I,C		I			C				I		T, E	
pET-45b(+)	●		●	N		C									E	

Vector amp<sup>R</sup> kan<sup>R</sup> T7lac His•Tag T7•Tag S•Tag Trx•Tag KSI HSV•Tag PKA Dsb•Tag GST•Tag Nus•Tag Strept•Tag II protease signal seq.

(continued on next page)

Vector	amp <sup>r</sup>	kan <sup>r</sup>	T7	T7lac	His•Tag <sup>®</sup>	T7•Tag <sup>®</sup>	S•Tag <sup>™</sup>	Trx•Tag <sup>™</sup>	KSI	HSV•Tag <sup>®</sup>	PKA	Dsb•Tag <sup>™</sup>	GST•Tag <sup>™</sup>	Nus•Tag <sup>™</sup>	Strep•Tag <sup>®</sup> /II	protease signal seq.
pET-46 Ek/LIC	●			●	N		C									E
pET-47b(+)		●		●	N		C									H, T
pET-48b(+)		●		●	I		C	N								H, T
pET-49b(+)		●		●	I		C						N			H, T
pET-50b(+)		●		●	N,I		C							I		H, T
pET-51b(+)	●			●	C										N	E
pET-51 Ek/LIC	●			●	C										N	E
pET-52b(+)	●			●	C										N	H, T
pET-52 3C/LIC	●			●	C										N	H, T

## Notes:

C: optional C-terminal tag      E: enterokinase H: HRV 3C      I: internal tag      LIC: ligation-independent cloning      N: N-terminal tag  
 signal seq.: signal sequence for potential periplasmic localization      T: thrombin      X: Factor Xa

## B. pET Vector Cloning Strategies

Many strategies can be used for subcloning an open reading frame into a pET vector for expression. Directional cloning can be accomplished with unique restriction sites in the multiple cloning region or through ligation-independent cloning (LIC) sites. The LIC method does not require restriction digestion or ligation and the LIC-prepared insert can be quickly cloned into multiple LIC vectors for high-throughput cloning. For maps of all of the pET vectors, please visit [www.novagen.com](http://www.novagen.com).

All of the pET translation vectors contain translation stop codons in all three reading frames following the cloning and tag regions as well as a downstream T7 transcription terminator. The terminator is not necessary for the efficient expression of most proteins, but note that some pET plasmids contain the gene for ampicillin resistance (*bla*) in the same orientation as the target gene. If the T7 transcription terminator is removed during cloning, IPTG-dependent accumulation of  $\beta$ -lactamase ( $M_r$  31.5 kDa) is usually observed along with the target protein, due to efficient read-through transcription by T7 RNA polymerase.

pET vectors contain different sequences adjacent to the cloning sites that encode a number of peptide “tags”, which facilitate localization, detection, or purification when fused with the target protein. The method of cloning will determine whether or not these “tags” or any additional amino acids from the vector are expressed fused to the protein of interest. The following sections describe several cloning options to produce target proteins with or without fusions.

### Produce recombinant proteins without fusions

Almost all of the pET vectors can express proteins that do not contain vector-encoded sequences. An *Nde* I or *Nco* I site is available in many vectors for cloning into the AUG start codon at the 5'-end of the insert coding sequence. Similarly, vector-encoded C-terminal fusions can be avoided by including a translation stop codon in the insert.

In many pET vectors, the ATG triplet within the *Nco* I site (CCATGG) encodes the N-terminal methionine AUG start codon in the T7 RNA polymerase transcripts. Target genes or PCR engineered inserts that contain either *Nco* I sites or sites that generate compatible overhangs [*Bsp*H I (TCATGA), *Bsp*LU11 I (ACATGT), and subsets of *Afl* III (ACRYGT) and *Sty* I (CCWWGG)] at the beginning of their ORF can be cloned into the *Nco* I site. However, utilization of these restriction sites can be complicated if the target gene encodes multiple internal sites. In addition, each of these restriction sites dictates the first nucleotide of the next triplet codon, which may prevent the generation of target protein without any extra amino acids. In such cases, it may be possible to use restriction enzymes that cleave “downstream” of their recognition site, to allow the generation of unfused target protein (see table below).

Enzyme (isoschizomers)	Recognition and cleavage site	Overhangs generated
<i>Bbs</i> I ( <i>Bpi</i> I, <i>Bpu</i> A I)	5' - GAAGAC (N) <sub>2</sub> - 3' 3' - CTTCTG (N) <sub>6</sub> - 5'	GAAGACNN      NNNNN CTTCTGNNNNNN      N
<i>Bsa</i> I ( <i>Eco</i> 31 I)	5' - GGTCTC (N) <sub>1</sub> - 3' 3' - CCAGAG (N) <sub>6</sub> - 5'	GGTCTCN      NNNNN CCAGAGNNNNN      N
<i>Bsm</i> B I ( <i>Esp</i> 3 I)	5' - CGTCTC (N) <sub>1</sub> - 3' 3' - GCAGAG (N) <sub>5</sub> - 5'	CGTCTCN      NNNNN GCAGAGNNNNN      N
<i>Bfu</i> A I	5' - ACCTGC (N) <sub>4</sub> - 3' 3' - TGGACG (N) <sub>8</sub> - 5'	ACCTGCNNNN      NNNNN TGGACGNNNNNNNN      N

Any of these restriction sites can be engineered into PCR primers such that *Nco* I-compatible overhangs can be generated. As with any strategy using restriction digestion, convenient utilization of this approach will be limited if the target gene encodes internal sites. However, it is relatively unlikely that a given insert will contain sites for all four of the enzymes listed above.

### Produce recombinant proteins without fusions after protease cleavage

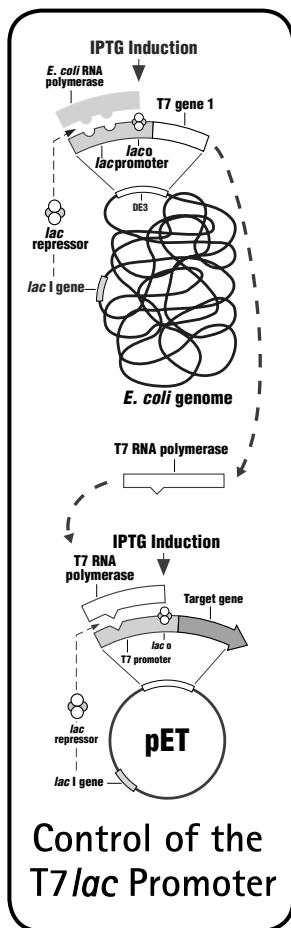
The pET-41a-c(+), 42a-c(+), pET-43.1a-c(+), pET-44a-c(+), pET-45b(+), pET-47b(+), pET-50b(+), and pET-52b(+) vectors contain *Psh*A I or *Sma* I restriction sites within sequences encoding Factor Xa, enterokinase, thrombin, or HRV 3C cleavage sites. Utilization of these blunt cutting restriction enzymes allows all (Factor Xa and enterokinase) or most (thrombin and HRV 3C) vector-encoded protein sequences to be removed from the resulting fusion proteins by cleavage with the appropriate protease. The efficiency of thrombin cleavage can be affected by the nature of the amino acids immediately following the cleavage site. Optimal thrombin cleavage is obtained when the first two to three insert-defined amino acids are apolar and non-acidic (Chang, 1985; Le Bonniec et al., 1991; Le Bonniec et al., 1996).

### Ligation-independent cloning

Ligation-independent cloning (LIC) was developed for the directional cloning of PCR products without restriction enzyme digestion or ligation reactions (Aslanidis et al., 1990, Haun et al., 1992). LIC-prepared pET vectors have non-complementary 12–15 base single-stranded overhangs that anneal to complementary single-stranded overhangs on the target insert. Primers amplifying the target insert require the addition of 5' extensions to create the complementary sequence to the prepared LIC vector. The 3'→5' exonuclease activity of T4 DNA polymerase produces the single stranded overhang on the insert during a short incubation. Cloning is directional, and is very fast and efficient because only the desired product is formed by annealing the prepared plasmid and insert. An additional

feature of the LIC vectors is the removal of all or most vector-encoded amino acids with the site specific proteases: enterokinase, Factor Xa, or HRV 3C. See User Protocols TB163, TB184, and TB453 for additional LIC strategy information.

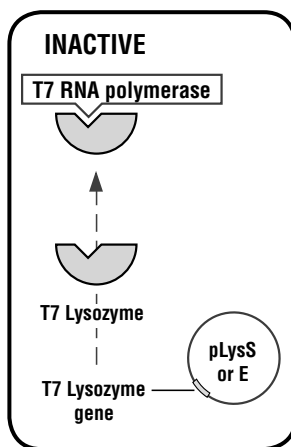
## C. Regulating Protein Expression in the pET System



Even in the absence of IPTG, there is some expression of T7 RNA polymerase from the *lacUV5* promoter in  $\lambda$ DE3 lysogens and therefore, basal expression of the target protein. Any recombinant protein expressed in *E. coli* may interfere with normal cell function and therefore may be “toxic” to the bacteria. The degree of toxicity will vary from protein to protein. If target gene products are sufficiently toxic to *E. coli*, this basal level of expression can be enough to prevent vigorous growth and the establishment of plasmids in  $\lambda$ DE3 lysogens. The pET System is a powerful protein expression tool because you can tightly control protein expression with the T7/T7lac promoter, pLysS or pLysE hosts, and addition of glucose to the media based on the characteristics of your target protein. It is possible to over-regulate the system with the result being low protein expression levels. Therefore it is important to both understand the following tools and empirically determine what combination is best suited for each protein of interest.

### The T7lac promoter

One approach to control basal expression is to use vectors that contain what is termed a T7lac promoter (Dubendorff et al., 1991; Studier et al., 1990; see table on page 42). These plasmids contain a *lac* operator sequence just downstream of the T7 promoter. They also carry the natural promoter and coding sequence for the *lac* repressor (*lacI*), oriented so that the T7lac and *lacI* promoters diverge. When this type of vector is used in DE3 lysogens, the *lac* repressor acts both at the *lacUV5* promoter in the host chromosome to repress transcription of the T7 RNA polymerase gene by the host polymerase and at the T7lac promoter in the vector to block transcription of the target gene by any T7 RNA polymerase that is made. Only a few target genes have been encountered that are too toxic to be stable in these vectors in expression host strains, such as BL21(DE3) or HMS174(DE3) (Dubendorff et al., 1991). Note that in combination with pLysS and pLysE hosts, expression can be over-regulated (see *Vector and host combinations affect expression levels* on page 46).



### pLysS and pLysE hosts

Additional stability target sequence stability can be achieved by expression in host strains containing a compatible chloramphenicol-resistant plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase (Moffatt et al., 1987; Studier, 1991). T7 lysozyme is a bifunctional protein: it cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall (Inouye et al., 1973), and it binds to T7 RNA polymerase, inhibiting transcription (Huang et al., 1999; X. Zhang et al., 1997). T7 lysozyme is provided by a clone of the T7 lysozyme gene in the *Bam*H I site of pACYC184 (Chang et al., 1978). The cloned fragment [bp 10,665–11,296 of T7 DNA; (Dunn et al., 1983)] also contains the  $\phi$ 3.8 promoter for T7 RNA polymerase immediately following the lysozyme gene. A plasmid having this fragment oriented so that the lysozyme gene is expressed from the *tet* promoter of pACYC184 is referred to as pLysE; cells carrying this plasmid

accumulate substantial levels of lysozyme. A plasmid having the fragment in the opposite orientation is referred to as pLysS; cells carrying this plasmid accumulate much lower levels of lysozyme. Note that expression of lysozyme from pLysS hosts is also dependent on culture conditions. Because the upstream chloramphenicol acetyl transferase (CAT) antibiotic resistance gene is regulated by a catabolite repression sensitive promoter, growing pLysS host strains to stationary phase in the absence of glucose can lead to high cAMP and higher CAT promoter activity. The higher CAT promoter activity may be the cause of elevated lysozyme levels observed in cultures grown to stationary phase (Novy et al., 2001). When produced from the cloned gene, relatively high levels of T7 lysozyme can be tolerated by *E. coli* (i.e., no cell lysis), apparently because the protein is unable to pass through the inner membrane to reach the peptidoglycan layer.

Neither lysozyme plasmid interferes with transformation of cells that contain it; pLysS has little effect on growth rate but pLysE causes a significant decrease in the growth rate of cells that carry it. The higher level of lysozyme provided by pLysE can substantially increase the lag time and reduce the maximum level of expression of target genes upon induction of T7 RNA polymerase. This damping effect on expression is sufficient that cells containing a target gene whose product is relatively innocuous can continue to grow indefinitely in the presence of IPTG, a property that may be useful in some circumstances. The presence of either pLysS or pLysE increases the tolerance of  $\lambda$ DE3 lysogens for plasmids with toxic inserts: unstable plasmids become stable, and plasmids that would not otherwise be established can be maintained and expressed. Because pLysE causes slower growth and a tendency toward lysis, its use is somewhat less convenient in most cases. For very toxic genes, the combination of a *T7lac* promoter-containing vector and pLysS is preferable.

The presence of pLysS or pLysE also makes it easier to prepare cell extracts. After the target protein has accumulated, the cells are collected and suspended in a buffer such as 50 mM Tris-HCl, 2 mM EDTA, pH 8.0. Simply freezing and thawing, or adding 0.1% Triton<sup>®</sup> X-100, will allow the resident T7 lysozyme to efficiently lyse the cells. PopCulture<sup>®</sup> and BugBuster<sup>®</sup> Protein Extraction Reagents release substantially more protein when used alone with hosts containing the pLysS or pLysE plasmids. This property can make it advantageous to carry pLysS in the cell even when it is not required for stabilizing the target plasmid. Note that the pLysS or pLysE plasmids are not recommended for use with constructs containing a signal sequence if isolation of the periplasmic fraction is desired (due to the breakdown of the cell membrane by the T7 lysozyme produced in those hosts).

### Vector and host combinations affect expression levels

In practice, it is usually worthwhile to test several different vector/host combinations to obtain the best possible yield of protein in its desired form. When the “plain” T7 promoter is used, the low level of lysozyme provided by pLysS has little effect on expression of target genes following induction of T7 RNA polymerase, except for a short lag in the appearance of target gene products. Apparently, more T7 RNA polymerase is induced than can be inhibited by the small amount of lysozyme. The level of lysozyme might be expected to increase somewhat upon induction, since T7 RNA polymerase should be able to transcribe completely around the pLysS plasmid from the  $\phi$ 3.8 promoter to make lysozyme mRNA. However, the  $\phi$ 3.8 promoter is relatively weak (McAllister et al., 1981), and most transcription should be from the much stronger  $\phi$ 10 promoter used in the target

plasmids. When using the *T7lac* promoter, we have observed that expression in pLysS hosts can be somewhat reduced relative to non-pLysS hosts under a given induction condition. For an example illustrating differences in the expression of two target proteins with various combinations of T7/T7lac promoter and pLysS and pLysE hosts, review Mierendorf et al. (1994).

### Media containing glucose

As first described by Grossman et al. (1998), low basal expression levels in the pET system can be maintained by supplementing the medium with glucose. As cultures reach stationary phase, any available glucose is consumed first and an alternative carbon source such as glycerol is then utilized. Metabolism of the alternate carbon source causes cyclic AMP (cAMP) levels to increase, stimulating transcription from the *lacUV5* promoter and subsequent expression of T7 RNA polymerase in  $\lambda$ DE3 lysogens. In contrast to the wild-type *lac* promoter, the *lacUV5* promoter is not as sensitive to cAMP stimulation (Eron et al., 1971; Fried et al., 1984). However, it has been demonstrated that sufficient stimulation occurs to elevate T7 RNA polymerase levels, and consequently, T7 promoter regulated target gene expression (Grossman et al., 1998; Kelley, 1995; Novy et al., 2001; Pan et al., 2000). A significant decrease in basal transcription from the *lacUV5* promoter is observed when standard medium is supplemented with glucose in cultures grown to stationary phase (Grossman et al., 1998; Novy et al., 2001; Pan et al., 2000).

Minimizing basal expression is particularly important for pET vector expression when hosts that do not carry the pLysS plasmid are allowed to grow to stationary phase (16 h; overnight cultures) and when the target gene is toxic (Grossman et al., 1998; Novy et al., 2001). Without the T7 lysozyme from the pLysS plasmid, basal expression levels are elevated in cultures grown to stationary phase. If the gene is toxic, the addition of 0.5–1% glucose to both liquid medium and agar plates may be necessary to maintain plasmid stability. Hosts containing pLysS may express an elevated level of lysozyme in cultures grown to stationary phase such that induced levels of the target protein are lowered. This is likely because the CAT gene promoter is also sensitive to stimulation by cAMP in the absence of glucose and is upstream of the T7 lysozyme gene in pLysS (Novy et al., 2001).

The addition of glucose is neither necessary nor recommended during the cloning steps in non-expression hosts. Although growing cultures to stationary phase is not recommended, glucose provides another method to maintain the lowest basal levels of target protein in  $\lambda$ DE3 lysogenic expression hosts used in the pET System and prevents overproduction of T7 lysozyme.

### pLacI hosts

The specialized (DE3)pLacI based expression hosts are only intended for use with the high copy number pETBlue™ and pTriEx™ vectors. These hosts supply *lac* repressor from the compatible pLacI plasmid to ensure stringent repression in the uninduced state. Host-provided *lac* repressor is required in pETBlue and pTriEx expression hosts because these plasmids do not contain the *lac* repressor gene. Refer to the pETBlue System Manual (User Protocol TB249) or the pTriEx System Manual (User Protocol TB250) for further details on use of pLacI hosts.

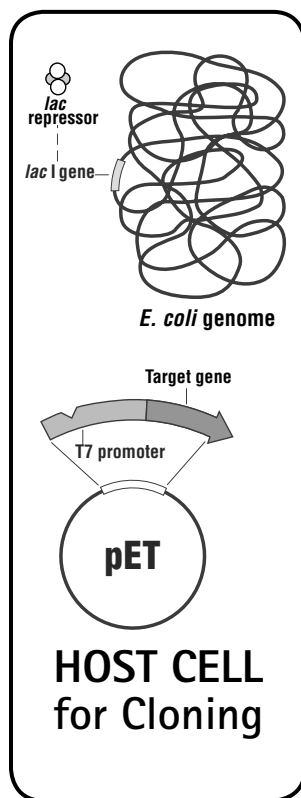
### pETcoco™ System

Another approach to minimize basal expression in  $\lambda$ DE3 lysogens is the use of the pETcoco vectors. These vectors are normally maintained as a single copy per cell, in contrast to the pET vectors which are maintained as approximately 40 copies per cell. When present as a single copy, target genes become extremely stable, both due to minimal opportunity for recombination or gene rearrangement, and reduction in basal transcription levels to about 1/40 of pET vectors. Protein expression of pETcoco recombinants is induced by IPTG, with expression levels comparable to pET vectors. For details see Sektas et al., 2002 and User Protocol TB333.

## D. Hosts for Cloning

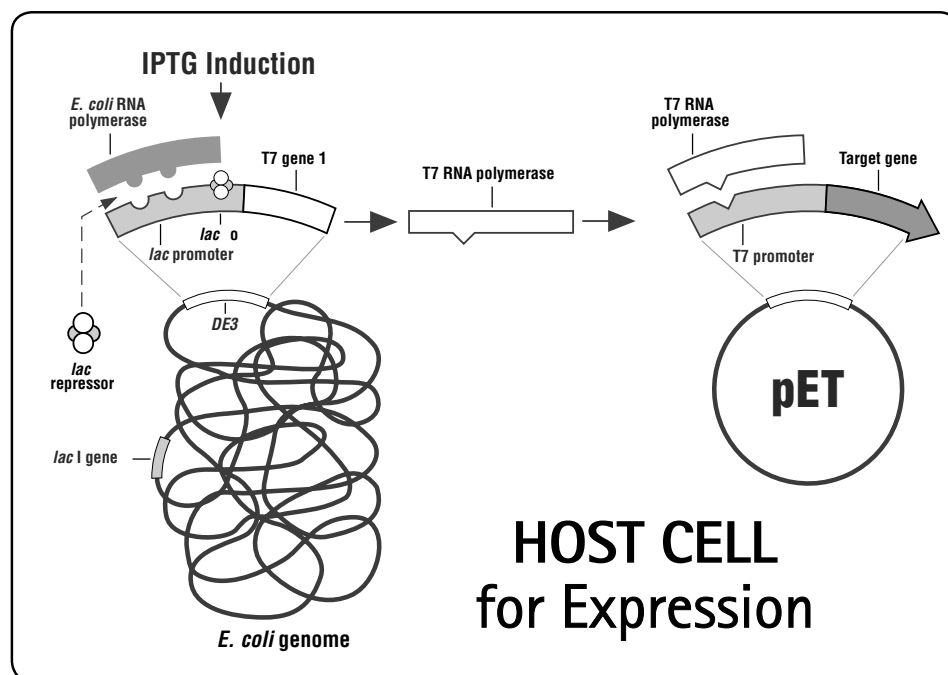
As described previously, a powerful feature of the pET system is the ability to clone target genes under conditions of extremely low transcriptional activity, that is, in the absence of a source of T7 RNA polymerase. Background expression is minimal in the absence of T7 RNA polymerase because the host RNA polymerases do not initiate from T7 promoters and the cloning sites in pET plasmids are in regions weakly transcribed (if at all) by read-through activity of bacterial RNA polymerase. Although in some cases (e.g., with innocuous target proteins), it may be possible to clone directly into expression hosts, this approach is not recommended as a general strategy. Even low levels of basal expression can cause growth problems and plasmid instability as a result of transcription from the T7 promoter.

Suitable bacterial hosts for cloning include the *E. coli* K12 strains NovaBlue, NovaBlue T1<sup>R</sup>, JM109, and DH5 $\alpha$ . These strains are convenient hosts for initial cloning of target DNA into pET vectors and for maintaining plasmids because they are *recA*<sup>-</sup> *endA*<sup>-</sup>, have high transformation efficiencies, and good plasmid yields. NovaBlue has the additional advantage of having a selectable F factor that allows helper phage infection and therefore the production of single-stranded plasmid DNA for mutagenesis purposes (appropriate only for plasmids carrying the f1 origin of replication). NovaBlue T1<sup>R</sup> have all the same features of NovaBlue cells, with the added benefit of being resistant to T1 and T5 phage. Note that there are no blue/white screening capabilities in the pET System because the pET vectors do not encode the *lacZ*  $\alpha$ -peptide. If blue/white screening in a T7 expression vector is required, the pETBlue plasmids provide this option in combination with NovaBlue (see User Protocol TB249). If desired, expression can be induced in the NovaBlue host or other non-DE3 hosts by infection with the bacteriophage  $\lambda$ CE6. See “*Bacteriophage CE6*” (page 19) for details.



## E. Hosts for Expression

For protein production, a recombinant plasmid is transferred to an *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase (T7 gene 1, see example below). These hosts are lysogens of bacteriophage DE3, a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase (Novy et al., 2001, Studier et al., 1986). This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter, which is inducible by addition of IPTG to the culture medium or by using an autoinduction medium (See Section IV, *Expressing the Target Gene*, page 19). DE3 lysogen strains may be chosen for protease deficiency, amino acid auxotrophy, solubility enhancement, rare codon supplementation, or other features. In addition, the  $\lambda$ DE3 Lysogenization Kit (Cat. No. 69734-3), allows the conversion of other *E. coli* strains to DE3 lysogens. Several popular commercial cloning vectors carry T7 promoters and separate *lac* operator/promoter elements for blue/white screening of recombinants. While in principle these vectors could be used with the pET expression hosts, these vectors are actually inappropriate for this purpose. The multiple copies of the *lac* operator on these plasmids will titrate *lac* repressor and partially induce the gene for T7 RNA polymerase in the pET host, which is also controlled by *lac* repressor. As a result, basal T7 RNA polymerase activity becomes high enough that many target genes cannot be stably maintained. These elements are properly balanced in the pETBlue™ System.



### Protease deficiency

All of the B strains (B834, BL21, BLR, Origami™ B, Rosetta™, Rosetta 2, Rosetta-gami™ B, and Tuner™) are deficient in the *lon* protease and lack the *ompT* outer membrane protease that can degrade proteins during purification (Grodberg et al., 1988). Thus, some target proteins may be more stable in these strains than in host strains containing these proteases. BL21(DE3) is the most widely used host for target gene expression. BLR(DE3) is a *recA*<sup>-</sup> derivative of BL21 constructed by A. Roca, University of Wisconsin, and may stabilize some target genes containing repetitive sequences. The Origami B, Rosetta, and Tuner strains are described in detail in the following sections.

### Adjustable expression levels throughout all cells in a culture

The Tuner™ strain and derivatives (Origami B and Rosetta-gami B) are *lacY1* deletion mutants of BL21 and enable adjustable levels of protein expression throughout all cells in a culture. The lac permease (*lacY1*) mutation allows uniform entry of IPTG into all cells in the population, which produces a concentration-dependent, homogenous level of induction. By adjusting the concentration of IPTG, expression can be regulated from very low level expression up to the robust, fully induced expression levels commonly associated with pET vectors. Lower level expression may enhance the solubility and activity of some target proteins.

### Disulfide bond formation and solubility enhancement

Many proteins require the formation of stable disulfide bonds for proper folding and activity. Without disulfide bonds, these proteins may be degraded or accumulate as inclusion bodies. One limitation of producing properly folded proteins in *E. coli* has been the relatively high reducing potential in the cytoplasmic compartment; disulfide bonds are usually formed only upon export into the periplasmic space. Bacterial strains with glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) mutations greatly enhance the formation of disulfide bonds in the *E. coli* cytoplasm (Aslund et al., 1999; Prinz et al., 1997).

The family of Origami strains carry the *trxB* and *gor* mutations for enhanced disulfide bond formation. Studies have shown that expression in Origami(DE3) yielded 10-fold more active protein than in another host, even though overall expression levels were similar (Prinz et al., 1997). If the target protein contains disulfide bonds and the target gene encodes rare codons, one of the Rosetta-gami strains may be optimal (see *Rare tRNA supplementation*, page 51). The Origami, Origami 2, Rosetta-gami, and Rosetta-gami 2 host strains are K-12 derivatives that carry the *trxB/gor* mutations. The Origami B and Rosetta-gami B host strains carry the same *trxB/gor* mutations as the original Origami strain, except that they are derived from *lacZY* mutant of BL21. The *trxB* and *gor* mutations in the Origami, Rosetta-gami, Origami B, and Rosetta-gami B host strains are selectable on kanamycin and tetracycline, respectively; therefore, these strains cannot be used with plasmid carrying kanamycin- or tetracycline-resistance genes. Unlike the original Origami strains, the Origami 2 and Rosetta-gami 2 strains are kanamycin sensitive, making these host strains compatible with many Novagen expression vectors. The *gor* mutation is still selected for by tetracycline, the same as the original strains. To reduce the possibility of disulfide bond formation between protein molecules, hosts containing the *trxB/gor* mutations are only recommended for the expression of proteins that require disulfide bond formation for proper folding.

### Rare tRNA supplementation

Most amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons. In each cell, the tRNA population closely reflects the codon bias of the mRNA population. When the mRNA of heterologous target genes is expressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. Insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshifting and amino acid misincorporation. The Rosetta™ strains are designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* (Brinkmann et al., 1989; Kane, 1995; Kurland et al., 1996; Seidel et al., 1992). By supplying rare tRNAs, the Rosetta strains provide for “universal” translation, where translation would otherwise be limited by the codon usage of *E. coli* (Brinkmann et al., 1989; Del Tito et al., 1995; Rosenberg et al., 1993; Seidel et al., 1992). Rosetta strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC and GGA on a compatible chloramphenicol-resistant plasmid. The Rosetta 2 strains supply a seventh rare codon (CGG) in addition to the six found in the original Rosetta strains. The tRNA genes are driven by their native promoters.

The Rosetta and Rosetta 2 strains are B-strain derivatives with *lon* and *ompT* protease deficiencies. The RosettaBlue strains are derived from the K-12 strain NovaBlue and have the added benefits of high transformation efficiency and stringency due to the high level of *lac* repressor (*lacI<sup>q</sup>*). The Rosetta-gami™ and Rosetta-gami 2 strains are K-12 strains with *trxB/gor* mutations, for enhanced protein folding through the formation of disulfide bonds (see *Disulfide bond formation and solubility enhancement*, page 50). The Rosetta-gami 2 strains are kanamycin sensitive and supplement seven rare codons. The Rosetta-gami B strains combine the key features of BL21 (and its Tuner™ derivative), Origami, and Rosetta to enhance both the expression of eukaryotic proteins and the formation of target protein disulfide bonds in the bacterial cytoplasm. In the pLysS and pLacI Rosetta strains, the rare tRNA genes are present on the same plasmids that carry the T7 lysozyme and *lac* repressor genes, respectively.

### Selenomethionine labeling

The B834 strain is a methionine auxotroph and the parental strain of BL21. B834 strains are useful for higher specific activity <sup>35</sup>S-met labeling and selenomethionine labeling for crystallography (Leahy, 1992; Wood, 1966). Significantly higher levels of several target proteins were achieved in B834(DE3) as compared to BL21(DE3), which suggests that there may be other advantages to using the parental strain (Doherty, 1995).

## pET system host strain characteristics table

This table lists the genotypes of strains commonly used for cloning and expression with the pET System. The catalog numbers for host strain glycerol stocks and competent cells can be found starting on page 73.

Strain	Deriv	Genotype	Description/Application	Antibiotic Resistance <sup>1</sup>
B834	B	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm met</i>	<i>met</i> auxotroph, parent of BL21, control non-expression <sup>2</sup> host	none
B834(DE3) <sup>9</sup>	B	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm met</i> (DE3)	<i>met</i> auxotroph, parent of BL21, general expression <sup>3</sup> host, <sup>35</sup> S-met labeling	none
B834(DE3)pLysS <sup>9</sup>	B	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm met</i> (DE3) pLysS (Cam <sup>R</sup> )	<i>met</i> auxotroph, parent of BL21, high-stringency expression <sup>3,4</sup> host, <sup>35</sup> S-met labeling	Chloramphenicol (34 µg/ml)
BL21	B834	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i>	control non-expression <sup>2</sup> host	none
BL21(DE3) <sup>9</sup>	B834	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3)	general purpose expression <sup>3</sup> host	none
BL21(DE3)pLysS <sup>9</sup>	B834	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3)pLysS (Cam <sup>R</sup> )	high-stringency expression <sup>3,4</sup>	Chloramphenicol (34 µg/ml)
BLR	BL21	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm Δ(<i>srl-recA</i>)306::Tn 10 (Tet<sup>R</sup>)</i>	<i>recA</i> <sup>-</sup> control non-expression <sup>2</sup> host recommended for use with tandem repeats	Tetracycline (12.5 µg/ml)
BLR(DE3) <sup>9</sup>	BL21	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3) Δ( <i>srl-recA</i> )306::Tn 10 (Tet <sup>R</sup> )	<i>recA</i> <sup>-</sup> expression <sup>3</sup> host recommended for use with tandem repeats	Tetracycline (12.5 µg/ml)
BLR(DE3)pLysS <sup>9</sup>	BL21	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3) Δ( <i>srl-recA</i> )306::Tn 10 pLysS (Cam <sup>R</sup> , Tet <sup>R</sup> )	<i>recA</i> <sup>-</sup> high-stringency expression <sup>3,4</sup> host recommended for use with tandem repeats	Chloramphenicol (34 µg/ml) Tetracycline (12.5 µg/ml)
HMS174	K-12	F <sup>-</sup> <i>recA1 hsdR(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>)</i> (Rif <sup>R</sup> )	control non-expression <sup>2</sup> host	Rifampicin (200 µg/ml)
HMS174(DE3) <sup>9</sup>	K-12	F <sup>-</sup> <i>recA1 hsdR(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>)</i> (DE3) (Rif <sup>R</sup> )	<i>recA</i> <sup>-</sup> K-12 expression <sup>3</sup> host	Rifampicin (200 µg/ml)
HMS174(DE3)pLysS <sup>9</sup>	K-12	F <sup>-</sup> <i>recA1 hsdR(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>)</i> (DE3) pLysS (Cam <sup>R</sup> , Rif <sup>R</sup> )	<i>recA</i> <sup>-</sup> K-12 high-stringency expression <sup>3,4</sup> host	Chloramphenicol (34 µg/ml) Rifampicin (200 µg/ml)
NovaBlue	K-12	<i>endA1 hsdR17(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>) supE44 thi-1 recA1 gyrA96 relA1 lac</i> F <sup>'</sup> [ <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> <i>lac</i> <sup>R</sup> <i>ZΔM15</i> ::Tn 10] (Tet <sup>R</sup> )	non-expression <sup>2</sup> host, general purpose cloning host, plasmid preps	Tetracycline (12.5 µg/ml)
NovaBlue(DE3)	K-12	<i>endA1 hsdR17(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>) supE44 thi-1 recA1 gyrA96 relA1 lac</i> (DE3) F <sup>'</sup> [ <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> <i>lac</i> <sup>R</sup> <i>ΔZ M15</i> ::Tn 10] (Tet <sup>R</sup> )	<i>recA</i> <sup>-</sup> <i>endA</i> <sup>-</sup> K-12 <i>lac</i> <sup>R</sup> expression <sup>3</sup> host recommended for use with NovaTope <sup>®</sup> System	Tetracycline (12.5 µg/ml)
NovaBlue T1 <sup>R</sup>	K-12	<i>endA1 hsdR17(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>) supE44 thi-1 recA1 gyrA96 relA1 lac tonA</i> F <sup>'</sup> [ <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> <i>lac</i> <sup>R</sup> <i>ΔZ M15</i> ::Tn 10] (Tet <sup>R</sup> )	non-expression <sup>2</sup> host, general purpose cloning, plasmid preps, T1 and T5 phage resistant	Tetracycline (12.5 µg/ml)
NovaXG	K-12	F <sup>-</sup> <i>mcrA Δ(mcrC-mrr) endA1 recA1 φ80dlacZ M15 lacX74 araD139 Δ(ara-leu)7697 galU galK rpsL nupG λ. tonA</i>	non-expression <sup>2</sup> host strain for demanding clonings. Methylation restriction minus. T1 and T5 phage resistant.	none
NovaXGF <sup>†</sup>	K-12	<i>mcrA Δ(mcrC-mrr) endA1 recA1 φ80dlacZ M15 lacX74 araD139 Δ(ara-leu)7697 galU galK rpsL nupG λ. tonA</i> F <sup>'</sup> [ <i>lac</i> <sup>R</sup> Tn 10] (Tet <sup>R</sup> )	non-expression <sup>2</sup> host strain for demanding clonings. Methylation restriction minus. T1 and T5 phage resistant.	Tetracycline (12.5 µg/ml)
Origami™ <sup>5</sup>	K-12	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL</i> F <sup>'</sup> [ <i>lac</i> <sup>+</sup> <i>lac</i> <sup>R</sup> <i>pro</i> ] <i>gor522</i> ::Tn 10 <i>trxB</i> (Kan <sup>R</sup> , Str <sup>R</sup> , Tet <sup>R</sup> )	control non-expression <sup>2</sup> host	Kanamycin (15 µg/ml) Streptomycin (50 µg/ml) <sup>9</sup> Tetracycline (12.5 µg/ml)
Origami(DE3) <sup>5</sup>	K-12	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL</i> F <sup>'</sup> [ <i>lac</i> <sup>+</sup> <i>lac</i> <sup>R</sup> <i>pro</i> ] (DE3) <i>gor522</i> ::Tn 10 <i>trxB</i> (Kan <sup>R</sup> , Str <sup>R</sup> , Tet <sup>R</sup> )	general expression <sup>3</sup> host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm	Kanamycin (15 µg/ml) Streptomycin (50 µg/ml) <sup>9</sup> Tetracycline (12.5 µg/ml)

(continued on next page; see footnotes on page 54)

Strain	Deriv	Genotype	Description/Application	Antibiotic Resistance <sup>1</sup>
Origami(DE3)pLysS <sup>5</sup>	K-12	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F' [lac<sup>+</sup> lacI<sup>h</sup> pro] (DE3) gor522 ::Tn 10 trxB pLysS (Cam<sup>R</sup>, Kan<sup>R</sup>, Str<sup>R</sup>, Tet<sup>R</sup>)</i>	high-stringency <sup>3,4</sup> expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm	Chloramphenicol (34 μg/ml) Kanamycin (15 μg/ml) Streptomycin (50 μg/ml) <sup>8</sup> Tetracycline (12.5 μg/ml)
Origami 2 <sup>5</sup>	K-12	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F' [lac<sup>+</sup> lacI<sup>h</sup> pro] gor522 ::Tn 10 trxB (Str<sup>R</sup>, Tet<sup>R</sup>)</i>	control non-expression <sup>2</sup> host; kanamycin sensitive	Streptomycin (50 μg/ml) <sup>8</sup> Tetracycline (12.5 μg/ml)
Origami 2(DE3) <sup>5</sup>	K-12	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F' [lac<sup>+</sup> lacI<sup>h</sup> pro] (DE3) gor522 ::Tn 10 trxB (Str<sup>R</sup>, Tet<sup>R</sup>)</i>	general expression <sup>3</sup> host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm; kanamycin sensitive	Streptomycin (50 μg/ml) <sup>8</sup> Tetracycline (12.5 μg/ml)
Origami 2(DE3)pLysS <sup>5</sup>	K-12	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F' [lac<sup>+</sup> lacI<sup>h</sup> pro] (DE3) gor522 ::Tn 10 trxB pLysS (Cam<sup>R</sup>, Str<sup>R</sup>, Tet<sup>R</sup>)</i>	high-stringency <sup>3,4</sup> expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm; kanamycin sensitive	Chloramphenicol (34 μg/ml) Streptomycin (50 μg/ml) <sup>8</sup> Tetracycline (12.5 μg/ml)
Origami™ B <sup>5</sup>	Tuner™ (B strain)	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1 ahpC gor522::Tn 10 trxB (Kan<sup>R</sup>, Tet<sup>R</sup>)</i>	control non-expression <sup>2</sup> host	Kanamycin (15 μg/ml) Tetracycline (12.5 μg/ml)
Origami B(DE3) <sup>5</sup>	Tuner (B strain)	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1 ahpC (DE3) gor522::Tn 10 trxB (Kan<sup>R</sup>, Tet<sup>R</sup>)</i>	general expression <sup>3</sup> host; contains Tuner <i>lac</i> permease mutation and <i>trxB/gor</i> mutations for cytoplasmic disulfide bond formation	Kanamycin (15 μg/ml) Tetracycline (12.5 μg/ml)
Origami B(DE3)pLysS <sup>5</sup>	Tuner (B strain)	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1 ahpC (DE3) gor522::Tn 10 trxB pLysS (Cam<sup>R</sup>, Kan<sup>R</sup>, Tet<sup>R</sup>)</i>	high-stringency <sup>3,4</sup> expression host; contains Tuner <i>lac</i> permease mutation and <i>trxB/gor</i> mutations for cytoplasmic disulfide bond formation	Chloramphenicol (34 μg/ml) Kanamycin (15 μg/ml) Tetracycline (12.5 μg/ml)
Rosetta™	BL21	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm pRARE<sup>6</sup> (Cam<sup>R</sup>)</i>	control non-expression <sup>2</sup> host	Chloramphenicol (34 μg/ml)
Rosetta(DE3) <sup>9</sup>	BL21	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm (DE3) pRARE<sup>6</sup> (Cam<sup>R</sup>)</i>	general expression <sup>3</sup> host; provides six rare codon tRNAs	Chloramphenicol (34 μg/ml)
Rosetta(DE3)pLysS <sup>9</sup>	BL21	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm (DE3) pLysSRARE<sup>6</sup> (Cam<sup>R</sup>)</i>	high-stringency <sup>3,4</sup> expression host; provides six rare codon tRNAs	Chloramphenicol (34 μg/ml)
Rosetta 2	BL21	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm pRARE2<sup>7</sup> (Cam<sup>R</sup>)</i>	control non-expression <sup>2</sup> host	Chloramphenicol (34 μg/ml)
Rosetta 2(DE3) <sup>9</sup>	BL21	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm (DE3) pRARE2<sup>7</sup> (Cam<sup>R</sup>)</i>	general expression <sup>3</sup> host; provides seven rare codon tRNAs	Chloramphenicol (34 μg/ml)
Rosetta 2(DE3)pLysS <sup>9</sup>	BL21	<i>F<sup>-</sup> ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm (DE3) pLysSRARE2<sup>6</sup> (Cam<sup>R</sup>)</i>	high-stringency <sup>3,4</sup> expression host; provides seven rare codon tRNAs	Chloramphenicol (34 μg/ml)
RosettaBlue™	K-12	<i>endA1 hsdR17(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>) supE44 thi-1 recA1 gyrA96 relA1 lac F' [proA<sup>+</sup> B<sup>+</sup> lacI<sup>h</sup> ΔZ M15 ::Tn 10] pRARE<sup>6</sup> (Cam<sup>R</sup>, Tet<sup>R</sup>)</i>	control non-expression <sup>2</sup> host	Chloramphenicol (34 μg/ml) Tetracycline (12.5 μg/ml)
RosettaBlue(DE3)	K-12	<i>endA1 hsdR17(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>) supE44 thi-1 recA1 gyr96 relA1 lac (DE3) F' [proA<sup>+</sup> B<sup>+</sup> lacI<sup>h</sup> ΔZ M15 ::Tn 10] pRARE<sup>6</sup> (Cam<sup>R</sup>, Tet<sup>R</sup>)</i>	<i>recA- endA- K-12 lacI<sup>h</sup></i> general expression <sup>3</sup> host; provides six rare codon tRNAs	Chloramphenicol (34 μg/ml) Tetracycline (12.5 μg/ml)
RosettaBlue(DE3) pLysS	K-12	<i>endA1 hsdR17(r<sub>K12</sub><sup>-</sup> m<sub>K12</sub><sup>+</sup>) supE44 thi-1 recA1 gyrA96 relA1 lac (DE3) F' [proA<sup>+</sup> B<sup>+</sup> lacI<sup>h</sup> ΔZ M15 ::Tn 10] pLysSRARE<sup>6</sup> (Cam<sup>R</sup>, Tet<sup>R</sup>)</i>	<i>recA- endA- K-12 lacI<sup>h</sup></i> high-stringency <sup>3,4</sup> expression host; provides six rare codon tRNAs	Chloramphenicol (34 μg/ml) Tetracycline (12.5 μg/ml)
Rosetta-gami™ <sup>5</sup>	Origami (K-12)	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F' [lac<sup>+</sup> lacI<sup>h</sup> pro] gor522 ::Tn 10 trxB pRARE<sup>6</sup> (Cam<sup>R</sup>, Kan<sup>R</sup>, Str<sup>R</sup>, Tet<sup>R</sup>)</i>	control non-expression <sup>2</sup> host	Chloramphenicol (34 μg/ml) Kanamycin (15 μg/ml) Streptomycin (50 μg/ml) <sup>8</sup> Tetracycline (12.5 μg/ml)
Rosetta-gami (DE3) <sup>5</sup>	Origami (K-12)	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F' [lac<sup>+</sup> lacI<sup>h</sup> pro] gor522 ::Tn 10 trxB pRARE<sup>6</sup> (Cam<sup>R</sup>, Kan<sup>R</sup>, Str<sup>R</sup>, Tet<sup>R</sup>)</i>	general expression <sup>3</sup> host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm, provides six rare codon tRNAs	Chloramphenicol (34 μg/ml) Kanamycin (15 μg/ml) Streptomycin (50 μg/ml) <sup>8</sup> Tetracycline (12.5 μg/ml)
Rosetta-gami™ (DE3)pLysS <sup>5</sup>	Origami (K-12)	<i>Δara-leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F' [lac<sup>+</sup> lacI<sup>h</sup> pro] gor522 ::Tn 10 trxB pLysSRARE<sup>6</sup> (Cam<sup>R</sup>, Kan<sup>R</sup>, Str<sup>R</sup>, Tet<sup>R</sup>)</i>	high-stringency <sup>3,4</sup> expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm, provides six rare codon tRNAs	Chloramphenicol (34 μg/ml) Kanamycin (15 μg/ml) Streptomycin (50 μg/ml) <sup>8</sup> Tetracycline (12.5 μg/ml)

(continued on next page; see footnotes on page 54)

Strain	Deriv	Genotype	Description/Application	Antibiotic Resistance <sup>1</sup>
Rosetta-gami™ 2 <sup>5</sup>	Origami (K-12)	$\Delta ara-leu7697 \Delta lacX74 \Delta phoA PvuII phoR araD139 ahpC galE galK rpsL$ F' [ <i>lac<sup>+</sup> lac<sup>R</sup> pro</i> ] <i>gor522::Tn 10 trxB</i> pRARE2 <sup>7</sup> (Cam <sup>R</sup> , Str <sup>R</sup> , Tet <sup>R</sup> )	control non-expression <sup>2</sup> host	Chloramphenicol (34 µg/ml) Streptomycin (50 µg/ml) <sup>8</sup> Tetracycline (12.5 µg/ml)
Rosetta-gami 2(DE3) <sup>5</sup>	Origami (K-12)	$\Delta ara-leu7697 \Delta lacX74 \Delta phoA PvuII phoR araD139 ahpC galE galK rpsL$ (DE3) F' [ <i>lac<sup>+</sup> lac<sup>R</sup> pro</i> ] <i>gor522::Tn 10 trxB</i> pRARE2 <sup>7</sup> (Cam <sup>R</sup> , Str <sup>R</sup> , Tet <sup>R</sup> )	general expression <sup>3</sup> host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm, provides seven rare codon tRNAs, kanamycin sensitive	Chloramphenicol (34 µg/ml) Streptomycin (50 µg/ml) <sup>8</sup> Tetracycline (12.5 µg/ml)
Rosetta-gami 2(DE3)pLysS <sup>5</sup>	Origami (K-12)	$\Delta ara-leu7697 \Delta lacX74 \Delta phoA PvuII phoR araD139 ahpC galE galK rpsL$ (DE3) F' [ <i>lac<sup>+</sup> lac<sup>R</sup> pro</i> ] <i>gor522::Tn 10 trxB</i> pLysSRARE2 <sup>7</sup> (Cam <sup>R</sup> , Str <sup>R</sup> , Tet <sup>R</sup> )	high-stringency <sup>3,4</sup> expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in <i>E. coli</i> cytoplasm, provides seven rare codon tRNAs; kanamycin sensitive	Chloramphenicol (34 µg/ml) Streptomycin (50 µg/ml) <sup>8</sup> Tetracycline (12.5 µg/ml)
Rosetta-gami B <sup>5</sup>	Tuner™ (B strain)	F - <i>ompT hsdSB(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1 ahpC gor522::Tn 10 trxB</i> pRARE (Cam <sup>R</sup> , Kan <sup>R</sup> , Tet <sup>R</sup> )	control non-expression <sup>2</sup> host	Chloramphenicol (34 µg/ml) Kanamycin (15 µg/ml) Tetracycline (12.5 µg/ml)
Rosetta-gami B(DE3) <sup>5</sup>	Tuner (B strain)	F - <i>ompT hsdSB(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1 ahpC</i> (DE3) <i>gor522::Tn 10 trxB</i> pRARE (Cam <sup>R</sup> , Kan <sup>R</sup> , Tet <sup>R</sup> )	general expression <sup>3</sup> host; contains Tuner <i>lac</i> permease mutation and <i>trxB/gor</i> mutations for cytoplasmic disulfide bond formation, provides six rare codon tRNAs	Chloramphenicol (34 µg/ml) Kanamycin (15 µg/ml) Tetracycline (12.5 µg/ml)
Rosetta-gami B(DE3)pLysS <sup>5</sup>	Tuner (B strain)	F - <i>ompT hsdSB(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1 ahpC</i> (DE3) <i>gor522::Tn 10 trxB</i> pLysSRARE (Cam <sup>R</sup> , Kan <sup>R</sup> , Tet <sup>R</sup> )	high-stringency <sup>3,4</sup> expression host; contains Tuner <i>lac</i> permease mutation and <i>trxB/gor</i> mutations for cytoplasmic disulfide bond formation, provides six rare tRNAs	Chloramphenicol (34 µg/ml) Kanamycin (15 µg/ml) Tetracycline (12.5 µg/ml)
Tuner™	BL21	F - <i>ompT hsdSB(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1</i>	control non-expression <sup>2</sup> host	none
Tuner(DE3)	BL21	F - <i>ompT hsdSB(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1</i> (DE3)	general expression <sup>3</sup> host; <i>lac</i> permease mutation allows control of expression level	none
Tuner(DE3)pLysS	BL21	F - <i>ompT hsdSB(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lacY1</i> pLysS (DE3) (Cam <sup>R</sup> )	high-stringency <sup>3,4</sup> expression host; <i>lac</i> permease mutation allows control of expression level	Chloramphenicol (34 µg/ml)

- The appropriate drug to select for the target plasmid must also be added.
- In this context, non-expression means that the strain does not contain the gene for T7 RNA polymerase and therefore will not express from target genes under the control of a T7 promoter. These strains may be suited for expression from *E. coli* promoters such as *lac*, *tac*, *trc*, and *trp*, or for infection by CE6 for pET expression.
- Expression means that the strain is a DE3 lysogen, i.e., it carries the gene for T7 RNA polymerase under *lacUV5* control. It is therefore suited for expression from T7 promoters.
- High-stringency means that the strain carries pLysS, a pET-compatible plasmid that produces T7 lysozyme, thereby reducing basal expression of target genes. pLysE hosts provide even greater stringency; these are available separately as glycerol stocks.
- The original *trxB/gor* double mutant (Stewart, 1998) required reducing agent in the growth medium to support normal growth rates. The Origami™ strains are a derivative (FA113) of the original strain that carry a mutation (*ahpC*) which allows normal growth rates in the absence of supplemental reducing agent (Bessette et al., 1999; Ritz et al., 2001).
- pRARE and pLysSRARE encode the tRNA genes *argU*, *araW*, *ileX*, *glyT*, *leuW*, *proL*, *metT*, *thrT*, *tyrU* and *thrU*. The rare codons AGG, AGA, AUA, CUA, CCC, and GGA are supplemented (Novy et al., 2001).
- pRARE2 and pLysSRARE2 contain the tRNA gene *argX* which recognizes the CGG codon for arginine in addition to tRNA genes supplied in the pRARE plasmid (see note 6).
- These strains carry a mutation in ribosomal protein (*rpsL*) conferring resistance to streptomycin; however streptomycin is not necessary to maintain strain genotype. If using pCDF vectors, spectinomycin must be used for antibiotic selection because *rpsL* mutation confers streptomycin resistance.
- These T7 expression strains produce functional *lac* permease and β-galactosidase and are therefore compatible with the Overnight Express™ Autoinduction Systems.

## VIII. Optimizing Expression

The following sections describe procedures and recommendations to optimize expression of your target protein. Included here are considerations for induction conditions, plasmid stability, protein solubility, and a discussion of factors that influence target gene expression.

### A. Induction Controls

An induction control strain that matches the type of promoter, selective marker, and other vector elements is included with each pET vector and expression system to allow convenient performance verification. The induction controls are not suitable for cloning. The strain is provided as a glycerol stock of an appropriate  $\lambda$ DE3 lysogen containing a pET plasmid with an insert encoding  $\beta$ -galactosidase, which can be easily assayed spectrophotometrically (except for Controls H, J, L, N, and O.1, which contain no insert). The following table lists the various induction control strains and matching pET vectors.

Control	Vector	Host strain	Selection	Promoter	Fusion tags	Protease site	Insert (protein size)	Included with vector/series	Cat. No.
A	pET-14b	BL21(DE3)pLysS	amp cam	T7	His•Tag®	T	$\beta$ -gal 118kDa	pET-3, 5, 12, 14b, 17b, 20b, 23	69674
B	pET-15b	BL21(DE3)pLysS	amp cam	T7lac	His•Tag	T	$\beta$ -gal 118kDa	pET-11, 15b, 21, 22b, 25b	69257
C	pET-16b	BL21(DE3)pLysS	amp cam	T7lac	His•Tag	X	$\beta$ -gal 119kDa	pET-16b	69675
D	pET-19b	BL21(DE3)pLysS	amp cam	T7lac	His•Tag	E	$\beta$ -gal 119kDa	pET-19b, 45b, 51b, 52b	69676
E	pET-28b(+)	BL21(DE3)	kan	T7lac	His•Tag T7•Tag®	T	$\beta$ -gal 119kDa	pET-9, 24, 26b, 27b, 28, 47b, 48b, 49b, 50b	69258
F	pET-29b(+)	BL21(DE3)	kan	T7lac	S•Tag™	T	$\beta$ -gal 119kDa	pET-29	69259
G	pET-30b(+)	BL21(DE3)	kan	T7lac	His•Tag S•Tag	T, E	$\beta$ -gal 121kDa	pET-30	69554
H	pET-31b(+)	BLR(DE3)pLysS	amp cam, tet	T7lac	KSI		none 14.8kDa	pET-31b	69966
J	pET-32a(+)	BL21(DE3)	amp	T7lac	Trx•Tag™ His•Tag S•Tag	T, E	none 20.4kDa	pET-32	69030
L	pET-39b(+)	BL21(DE3)	kan	T7lac	DsbA•Tag™ His•Tag S•Tag	T, E	none 32.2kDa	pET-39b, 40b	70463
M	pET-33b(+)	BL21(DE3)	kan	T7lac	His•Tag PKA site T7•Tag	T	$\beta$ -gal 120kDa	pET-33b	70514
N	pET-41b(+)	BL21(DE3)	kan	T7lac	GST•Tag™ His•Tag S•Tag	T, E	none 35.6kDa	pET-41, 42	70535
O.1*	pET-43.1b(+)	BL21(DE3)	amp	T7lac	Nus•Tag™ His•Tag S•Tag HSV•Tag®	T, E	none 66.4kDa	pET-43.1, 44	70965

#### Abbreviations:

amp; ampicillin or carbenicillin    cam; chloramphenicol    E; enterokinase    kan; kanamycin  
tet; tetracycline    T; thrombin    X; Factor Xa

\* Induction control O (70833-3) has identical characteristics as O.1 with the exception that pET-43b(+) was provided.

The provided induction control can be used to verify the performance for bacterial expression and affinity purification under both native and denaturing conditions. Details of the plasmid constructs in the control strains are provided in the table on the previous page.

### $\beta$ -galactosidase assay

Many of the induction controls express *E. coli*  $\beta$ -galactosidase ( $\beta$ -gal) as the target gene, enzymatic activity can be used to easily follow the protein through the purification and cleavage steps. The BetaRed™  $\beta$ -Galactosidase Assay Kit (Cat. No. 70978-3) provides a rapid, sensitive measurement of  $\beta$ -galactosidase activity in cell extracts. Extracts prepared with BugBuster® Reagent, PopCulture® Reagent, or standard PBS and Tris-based lysis buffers are compatible with this assay as well as rLysozyme™ Solution and Benzonase® Nuclease. The colorimetric BetaRed Assay is 10-fold more sensitive (1 pg) than ONPG-based assays. For a detailed protocol, see User Protocol TB303.

## B. Enhancing Solubility and Folding

Recombinant proteins expressed in *E. coli* are often produced as aggregates called inclusion bodies. Even when inclusion bodies are formed, some proportion of the target protein is usually soluble within the cell. With the high expression levels of the pET System there may be a significant amount of soluble material even when most of the target protein mass is aggregated. In general, conditions that decrease the rate of protein synthesis, such as low induction temperatures or growth in minimal media, tend to increase the percentage of target protein found in soluble form.

In many applications, it is desirable to express target proteins in their soluble, active form. The following sections describe several suggestions to enhance solubility of the target protein. It should be noted that solubility does not necessarily indicate that a protein is folded properly; some proteins form soluble species that are inactive. The vector, host, protein sequence, and culture conditions can contribute to either increase or decrease the proportion of soluble and insoluble forms.

### Temperature

Growth at 37°C causes some proteins to accumulate as inclusion bodies, while incubation at 30°C may lead to soluble, active protein (Schein et al., 1989). Growth and induction at 25°C or 30°C may be optimal if you want to export the target using the signal sequence leaders present in a number of pET vectors. In some cases, prolonged (e.g., overnight) induction at low temperatures (15°–20°C) may prove optimal for the yield of soluble protein.

### Lysis buffer

The partitioning of a given target protein into the soluble or insoluble fraction can be strongly influenced by the nature of the lysis buffer. Proteins containing hydrophobic or membrane-associated domains may partition into the insoluble fraction when using a standard lysis buffer, such as 1X His•Bind® Binding Buffer (which contains 500 mM NaCl), but may not actually be present in inclusion bodies. Proteins in the insoluble

fraction due to association with bacterial lipids or membranes may often be converted to the soluble fraction by adding millimolar amounts of nonionic or zwitterionic detergents to the lysis buffer. BugBuster® Protein Extraction Reagent or PopCulture® Reagent, both used in combination with rLysozyme™ Solution, can be an effective choice to consider for increased recovery of lipid or membrane associated proteins in the soluble fraction. The proprietary formulations utilize a nonionic and zwitterionic detergent cocktail capable of solubilizing cell wall and membrane components, thereby releasing cellular proteins without denaturation. The detergents in BugBuster and PopCulture reagents will not facilitate solubilization of all membrane bound or hydrophobic proteins. Other detergents may be necessary to solubilize these membrane proteins and it is possible some may not be solubilized. Choosing a detergent for solubilization remains an empirical task. For a review of the use of detergents in bacterial lysis, see “Experiment 2: Solubilization and Purification of the Rat Liver Insulin Receptor” (Brennan et al., 1996). Note, however, that the addition of detergent may affect downstream purification procedures.

Target proteins that contain highly charged domains may also associate with other cellular components (e.g., basic proteins may bind to DNA). In these cases, the target protein may partition with cellular debris; in theory, they may be dissociated by adding salt to the lysis buffer or digesting the nucleic acid with a nuclease such as Benzonase® Nuclease (see User Protocol TB261).

### Periplasmic localization

An alternative strategy to obtain active, soluble proteins is to use vectors that enable export into the periplasm, which is a more favorable environment for folding and disulfide bond formation (Raina et al., 1997; Rietsch et al., 1996; Sone et al., 1997). For this purpose vectors carrying signal peptides are used, such as pET-20, 22, 25, 26, 27, 39, and 40. However, some target proteins will not be good candidates for periplasmic localization. For example, some fusions of  $\beta$ -gal to a periplasmic protein have proven to be toxic (Snyder et al., 1995). In addition, the net charge of the N-terminal amino acids on the mature protein can inhibit translocation (Kajava et al., 2000).

While several pET vectors contain signal sequences for fusion with target genes, pET-39b(+) and pET-40b(+) are designed to create fusions to the enzymes that catalyze the formation (DsbA) or isomerization (DsbC) of disulfide bonds in the periplasm (Missiakas et al., 1994; Zapun et al., 1995). If a fusion protein is competent to localize to the periplasm, then its direct association with the catalytic enzyme may enhance its solubility and facilitate disulfide bond formation. A properly folded fusion protein requiring formation of disulfide bonds for activity has been isolated following fusion to DsbA (Collins-Racie et al., 1995). Note that overexpressed, purified DsbC enzyme is isolated in the oxidized state and requires exposure to a reducing agent (0.1 to 1.0 mM DTT) to acquire disulfide isomerase activity *in vitro* (Joly et al., 1997). Typically, a DsbC fusion protein expressed from pET-40b(+) is first purified by His•Bind® or Ni-NTA His•Bind chromatography. Prior to exposing the fusion protein to a reducing agent, either EDTA should be added to a final concentration of 1 mM, or the sample should be dialyzed to remove residual Ni<sup>2+</sup>. EDTA addition and dialysis are probably unnecessary if Ni-NTA His•Bind resin was used for purification.

## Cytoplasmic localization

The Trx•Tag<sup>™</sup>, GST•Tag<sup>™</sup>, and Nus•Tag<sup>™</sup> fusion tags are highly soluble polypeptides that can potentially enhance solubility of target proteins. When using vectors designed for cytoplasmic expression, folding can be improved in hosts that are permissive for the formation of disulfide bonds in the *E. coli* cytoplasm (see *Host Strains*, below). Recognition sequences for site specific proteases are engineered into these vectors for complete removal of the tags (see page 42).

Schistosomal glutathione-S-transferase (GST) is commonly used as an N-terminal fusion partner when expressing proteins in *E. coli*. Although not specifically designed for this purpose, the GST•Tag sequence has been reported to enhance the solubility of its fusion partners. The pET-41a-c(+), pET-42a-c(+), and pET-49b(+) vectors encode the GST•Tag sequence that can be cleaved with thrombin or enterokinase, Factor Xa, and HRV 3C, respectively.

Many proteins that are normally produced in an insoluble form in *E. coli* tend to become more soluble when fused with the N-terminal thioredoxin (Trx•Tag) sequence (LaVallie et al., 1993; Novy et al., 1995). The Trx•Tag expressed from pET-32a-c(+) and pET-48b(+) vectors not only enhances the solubility of many target proteins, but appears to catalyze the formation of disulfide bonds in the cytoplasm of *trxB* mutants (Stewart, 1998). The combination of Trx•Tag containing vectors and *trxB/gor* mutant strains that promote disulfide bond formation in the cytoplasm may yield maximum levels of soluble, active, properly folded target proteins.

The pET-43.1a-c(+), pET-44a-c(+), and pET-50b(+) vectors incorporate a solubility-promoting peptide, the Nus•Tag<sup>™</sup> sequence, which was developed through a systematic search for *E. coli* proteins that have the highest potential for solubility when overexpressed (Davis et al., 1999; Harrison, 2000). Greater than 85% of the expressed protein was soluble in tests with each of four different NusA fusion proteins (Harrison, 2000). pET-43.1, 44, and 50 vectors are also compatible with the *trxB/gor* mutant strains (see below).

## Host strains

Many proteins require disulfide bonds for proper folding and activity; however, the cytoplasm of *E. coli* is not a favorable environment for disulfide bond formation. The use of Origami<sup>™</sup>, Origami 2, Origami B, Rosetta-gami<sup>™</sup>, Rosetta-gami 2, or Rosetta-gami B host strains promote the formation of disulfide bonds in the *E. coli* cytoplasm, which may affect the solubility and/or activity of a given target protein. If the target protein contains disulfide bonds and the target gene encodes rare codons, one of the Rosetta-gami strains may be optimal (see below, *Correcting for rare codon usage*). If your target protein contains one or more essential disulfide bonds, the combination of a Trx•Tag containing vectors and *trxB/gor* host may prove to be optimal because disulfide bond formation in the cytoplasm appears to be dependent on the presence of thioredoxin (Stewart, 1998). Also review *Disulfide bond formation and solubility enhancement* on page 50.

The Tuner™ strain and its derivatives (Origami™ B and Rosetta-gami B) are *lacY1* deletion mutants of BL21 and enable adjustable levels of protein expression throughout all cells in a culture. By adjusting the concentration of IPTG, expression can be regulated from very low level expression up to the robust, fully induced expression levels commonly associated with pET vectors. Lower level expression may enhance the solubility and activity of difficult target proteins.

## C. Correcting for Rare Codon Usage

Most amino acids are encoded by more than one codon, and analysis of *E. coli* codon usage reveals that several codons are underrepresented. The tRNA population closely reflects the codon bias of the mRNA population and when the mRNA of heterologous target genes is overexpressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. Insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshifting, and amino acid misincorporation.

Although the presence of a small number of rare codons often does not severely depress target protein synthesis, heterologous protein expression can be very low when a gene encodes clusters of and/or numerous rare *E. coli* codons. Excessive rare codon usage in the target gene has been implicated as a cause for low level expression (Sorensen et al., 1989; Zhang et al., 1991) as well as truncation products. The effect seems to be most severe when multiple rare codons occur near the amino terminus (Chen et al., 1990). A number of studies have indicated that high usage of the arginine codons AGA and AGG can have severe effects on protein yield. The impact appears to be highest when these codons are present near the N-terminus and when they appear consecutively (Brinkmann et al., 1989; Calderone et al., 1996; Hua et al., 1994; Schenk et al., 1995; Zahn, 1996). Several laboratories have shown that the yield of protein whose genes contain rare codons can be dramatically improved when the cognate tRNA is increased within the host (Brinkmann et al., 1989; Rosenberg et al., 1993; Seidel et al., 1992). For example, the yield of human plasminogen activator was increased approximately 10-fold in a strain that carried an extra copy of the tRNA for AGG and AGA on a compatible plasmid (Brinkmann et al., 1989). Increasing other rare tRNAs for AUA, CUA, CCC, or GGA have all been used to augment the yield and fidelity of heterologous proteins (Kane, 1995).

The Rosetta™ strains supplement tRNAs rarely utilized in *E. coli* on a chloramphenicol resistant plasmid (pACYC backbone) compatible with pET vectors. For more information on these strains see *Rare codon supplementation*, page 51.

## D. Toxic Genes and Plasmid Instability

Plasmid pBR322 and many of its derivatives (including pET vectors) are relatively stable and are retained by a very high fraction of host cells even after growth for many generations in the absence of a selective antibiotic. However, problems of plasmid instability can arise when a gene whose product is toxic to the host cell is cloned in the plasmid. The pETcoco™ System reduces background expression to the lowest levels by reducing the copy number of the pETcoco plasmid to one copy per cell (Sektas et al., 2002). The most toxic gene products may be stabilized and expressed in this system (see User Protocol TB333).

In the pET System, the level of expression may be such that the plasmid can be maintained but growth of the cell is impaired; segregation of cells lacking plasmid may also be increased because of decreased copy number or for other reasons. In such a situation, cells that lack the plasmid can rapidly overgrow the culture whenever selective antibiotic is lacking. If the plasmid is to be maintained in a significant fraction of the cells, the culture must not be allowed to grow in the absence of selection for the plasmid. The following sections describe several options for increasing plasmid stability.

### Use of ampicillin

Use of ampicillin as a selective antibiotic requires special care because  $\beta$ -lactamase is made in substantial amounts and is secreted into the medium, where it can destroy all of the ampicillin. In addition, ampicillin is susceptible to hydrolysis under acidic media conditions created by bacterial metabolism. This means that a culture in which the cells carry an unstable plasmid will be growing under ampicillin selection only until enough  $\beta$ -lactamase has been secreted to destroy the ampicillin in the medium. From that point on, cells that lack plasmid will not be killed and will begin to overgrow the culture. For a typical pBR322-based plasmid growing in a medium containing 50  $\mu$ g ampicillin per milliliter, this point is reached when the culture is barely becoming turbid (about  $10^7$  cells per milliliter). The presence of 200  $\mu$ g ampicillin per milliliter delays this point to a slightly higher cell density, but given the catalytic activity of  $\beta$ -lactamase, it would not be feasible to add enough ampicillin to the medium to keep the cells under selection all the way to saturation.

A further complication is that certain toxic genes kill cells at saturation, while having little effect on cells that are growing logarithmically. Almost all cells retain plasmid until saturation, but upon continued incubation, fewer and fewer plasmid-containing cells survive and, because no ampicillin remains, cells that lack plasmid overgrow the culture.

A culture grown to saturation from selective conditions will have secreted a considerable amount of  $\beta$ -lactamase into the medium, even if it becomes substantially overgrown by cells that lack plasmid. Subcultures might typically be grown from dilutions of 200- to 1000-fold into fresh ampicillin-containing medium. However, enough  $\beta$ -lactamase is typically present in the saturated culture that, even at these dilutions, enough remains to destroy all of the ampicillin before the cells that lack plasmid can be killed. Therefore, the subculture will grow completely in the absence of selection. The inoculum may already have had a substantial fraction of cells lacking plasmid, and by the time the subculture has grown to a density where expression of the target gene is to be induced, possibly only a minor fraction of the cells will contain the target plasmid. Failure to appreciate these

potential problems can easily lead to the erroneous conclusion that certain target genes are poorly expressed, when in fact only a small fraction of cells in the cultures that were tested contained plasmid.

Simple precautions are recommended to maximize retention of plasmid through the procedures for isolating, maintaining, and expressing target plasmids. Experiments at Novagen scientists have shown that the use of carbenicillin in place of ampicillin helps prevent overgrowth of cultures by cells that have lost the plasmid, partially due to its superior stability at low pH. Another alternative is to choose a pET vector containing the kanamycin resistance marker instead of the *bla* gene. Also, avoid growing the amp<sup>R</sup> cultures into saturation phase (overnight; 16 h) to maintain maximum selection. A more detailed discussion of the potential advantages of kan<sup>R</sup> compared to amp<sup>R</sup> is presented in *Antibiotic Resistance*, page 40.

### Supplementing with glucose

Plasmids containing toxic genes may be destabilized in  $\lambda$ DE3 lysogens during overnight cultures due to cAMP stimulation of T7 RNA polymerase (Grossman et al., 1998). This can be effectively eliminated by avoiding overnight cultures or delayed by including 0.5–1% glucose in the culture medium. Review *Media containing glucose*, page 47.

### Stabilize a toxic gene in an amp<sup>R</sup> pET vector for glycerol stock storage

The following protocol usually produces the highest possible fraction of cells containing functional ampicillin-resistant target plasmid.

#### Storage of ampicillin-resistant strains:

1. Inoculate a colony from the transformation plate into 2 ml LB + 50  $\mu$ g/ml carbenicillin and incubate for a few hours, until the culture becomes slightly turbid.
2. Streak a sample on a plate containing carbenicillin to obtain a single colony.

*Tip:* If the target gene is believed to be highly toxic, streak on LB agar plates containing 0.5–1% glucose to help reduce basal expression levels.

3. As soon as the colony develops (usually overnight at 37°C), inoculate into 2 ml LB + 50  $\mu$ g/ml carbenicillin and grow until OD<sub>600</sub> = 0.5.
4. Mix 0.9 ml of culture with 0.1 ml of 80% glycerol in a cryovial and store at –70°C.

#### Stabilize a toxic gene in amp<sup>R</sup> pET vector during induction

The following induction protocol has been successfully used with an extremely toxic gene in pET-22b(+). It involves the use of a high concentration of carbenicillin and replacing the medium twice prior to induction.

#### Induction of toxic genes:

1. Inoculate a single colony into 2 ml TB + 200  $\mu$ g/ml carbenicillin. Grow the cells at 37°C until OD<sub>600</sub> = 0.2–0.6.
2. Collect the cells by centrifugation (30 s in a microcentrifuge), remove the supernatant

and resuspend in 2 ml fresh media. Add a 100 µl sample to 8 ml TB + 500 µg/ml carbenicillin and grow the culture at 37°C until  $OD_{600} = 0.2-0.6$ .

*Note:* The removal of old medium removes the secreted  $\beta$ -lactamase.

3. Collect the cells by centrifugation at  $1000 \times g$  for 5 min and resuspend in fresh TB + 500 µg/ml carbenicillin containing 1 mM IPTG. Incubate at 30°C for 2 h before harvest.

## E. Coexpression of target proteins

Coexpression of multiple target genes in *E. coli* has been demonstrated to enhance yield, solubility, and activity of proteins that either make up part of a multi-protein complex or require coexpression with chaperones. Coexpression can also greatly facilitate the analysis of multi-subunit complexes and biochemical pathways and the characterization of protein-protein interactions, among other applications (Novy, et al., 2002).

Coexpression of multiple target genes in *E. coli* can be achieved by either cloning and expressing two or more open reading frames (ORFs) in a single vector or by transforming cells with two or more plasmids with compatible replicons and different drug resistance genes.

### Coexpression vectors and adaptors

The following T7 promoter-based vectors-adaptors are available for coexpression of multiple target in *E. coli*:

- The Duet system consists of five vectors, each of which is capable of coexpressing two target proteins or, when transformed with one another, or with other pET vectors, coexpressing up to eight proteins in one cell in *E. coli* when using appropriate host strains. For more information on the Duet system, see User Protocol TB340.
- The pCDF and pRSF vectors have different replicons and drug resistance genes. These two features make the pCDF-1b and pRSF-1b vectors suitable for coexpression with each other as well as with many other pET vectors. For more information on pCDF and pRSF vectors, see User Protocol TB401.
- The pETcoco™ System consists of two vectors that are compatible with many expression vectors and have the added benefit of allowing control over the number of copies present per cell for cloning and expression purposes. For more information on the pETcoco System, see User Protocol TB333.
- The LIC Duet™ Adaptors make any pET, pRSF, or pCDF Ek/LIC-prepared plasmid a coexpression vector. The adaptors are designed to facilitate the simultaneous cloning of two ORFs into one plasmid and their subsequent coexpression in *E. coli*. Five adaptors are available, four of which encode fusion tags that aid in purification and/or may enhance solubility of the target protein. The fifth is a “mini” adaptor for minimal vector-encoded fusion sequences. For more information on the LIC Duet Adaptors, see User Protocol TB384.

## Replicons, drug resistance, and copy number

Vectors used for coexpression must have compatible replicons and different drug resistance markers. The table below summarizes the replicons, copy number, and compatibility of the Novagen vectors recommended for coexpression in *E. coli*.

Plasmid(s)	Replicon (source)	Copy number*	Compatible Replicons
pET (all), pETDuet-1	ColE1 (pBR322)	~40	P15A, Mini-F/RK2, CloDF13, RSF1030, ColA
pACYCDuet-1, pLysS, pLysE, pLacI, pRARE, pRARE-2	P15A (pACYC184)	10–12	ColE1, Mini-F/RK2, CloDF13, RSF1030, ColA
pCDFDuet™-1, pCDF	CloDF13	20–40	ColE1, P15A, RSF1030, ColA
pRSFDuet-1, pRSF	RSF1030	> 100	ColE1, P15A, CloDF13
pCOLADuet™-1	COLA (ColA)	20–40	ColE1, P15A, CloDF13, Mini-F/RK2
pETcoco™ (all)	Mini-F/RK2 (pBeloBAC11, RK2)	amplifiable to ~40	ColE1, P15A, ColA

\* Copy number was estimated based on gel analysis (Held, et. al, 2003; Sektas, M. and Szybalski, W. 2002)

## F. Other Factors Influencing Expression

This T7 expression system has been used to produce substantial amounts of target protein from a wide variety of genes, both prokaryotic and eukaryotic. However, a few proteins are expressed in disappointingly small amounts, for reasons that are obvious in some cases and obscure in others. The target protein itself may interfere with gene expression or with the integrity of the cell. Sometimes pulse labeling shows a gradual or rapid decrease in the rate of protein synthesis as target protein accumulates, or sometimes all protein synthesis stops before any target protein can be detected. Occasionally, considerable lysis of a culture is observed. The following sections briefly summarize some of the known or likely reasons for obtaining low levels of expression and describe recommendations for optimizing protein expression.

### N-end rule

Another factor that appears to influence target protein stability is the amino acid immediately following the N-terminal methionine (penultimate amino acid). The amino acid at this position determines the removal of N-terminal fMet. Processing is catalyzed by methionyl aminopeptidase and is governed by the following relationship: the degree of removal decreases as the size of the penultimate amino acid side chain increases (Hirel et al., 1989; Lathrop et al., 1992). In practice, little or no processing was observed by these authors when the following amino acids occupied the penultimate position: His, Gln, Glu, Phe, Met, Lys, Tyr, Trp, or Arg. Processing ranged from 16–97% when the remaining amino acids occupied this position.

The relationship between a protein's amino terminal amino acid and its stability in bacteria is determined by the N-end rule (Tobias et al., 1991). They reported protein half-lives of only 2 minutes when the following amino acids were present at the amino terminus: Arg, Lys, Phe, Leu, Trp, or Tyr. In contrast, all other amino acids conferred half-lives of greater than 10 hours when present at the amino terminus in the protein examined.

Taken together, these studies suggest that Leu in the penultimate position would be a poor choice, because it would likely be exposed by fMet processing and then targeted for rapid degradation. Therefore, when a *Nde* I site is employed for the production of unfused target proteins from pET vectors, Leu codons in the penultimate position should be avoided. Leu codons in this position are not available when using *Nco* I as the cloning site, because the penultimate codon must begin with G.

### Secondary site translation initiation

Occasionally, truncated expression products are observed in addition to full-length target proteins. One obvious explanation is proteolytic degradation; however, secondary site translation initiation is another possibility (Halling et al., 1985; Preibisch et al., 1988). This can occur within an RNA coding sequence when a sequence resembling the ribosome binding site (AAGGAGG) occurs with the appropriate spacing (typically 5–13 nucleotides) upstream of an AUG (Met) codon. These truncated products can be problematic when attempting to purify full-length proteins. One possible solution is to employ pET vectors that allow fusion to affinity tags at both ends of the target protein. Several pET vector series enable His•Tag® fusions at both the N- and C-terminus. Full-length proteins would then be expected to elute at higher imidazole concentrations than truncated forms. Other pET vectors enable a combination of different tags to be used at each end of the target protein, e.g., T7•Tag®, S•Tag™, Strep•Tag® II and/or GST•Tag™ N-terminal fusion and His•Tag C-terminal fusion. Performing sequential affinity purification can isolate the full-length target protein.

### Secondary structure in the mRNA transcript

Secondary structure in the mRNA transcript can interfere with the AUG translation initiation codon and/or the ribosome binding site (Lee et al., 1987; Looman et al., 1986; Tessier et al., 1984). All pET vectors will generate one of the following transcripts:

rbs	<i>Nde</i> I/ <i>Nco</i> I
5'..AAGAAGGAGAUUAUCAUAUG...3'	
5'..AAGAAGGAGAUUAACCAUGG...3'	

If poor expression is observed, searching the coding strand of an insert for stretches of complementarity with the above sequences (i.e., 5'-CATATGTATATCTCCTTCTT-3', or 5'-CCATGGTATATCTCCTTCTT-3') may reveal whether secondary structure is a potential problem.

### Unexpected stop codons

Unexpected stop codons can be generated by mutation, especially when cloning PCR products. Sequencing can reveal these mutations, but another alternative is to test the construct's ability to produce the target protein by *in vitro* translation.

### Transcription terminator

Many target proteins seem to be made in equivalent amounts whether or not the T $\phi$  transcription terminator is present in the vector. In some cases, however, having T $\phi$  behind the target gene increases the production of target protein; this has been found when the target gene carries its own translation initiation signals (Studier et al., 1990). A possible interpretation is that some translation initiation signals do not compete well against the *bla* mRNA, which is made along with the target mRNA in many amp<sup>R</sup> pET vectors. Because T $\phi$  reduces the amount of this competing mRNA, it allows more target protein to be made. In all the kan<sup>R</sup> pET vectors and the latest amp<sup>R</sup> pET vectors (see page 41), the kan or amp genes and the target gene have opposite orientations so no competing mRNAs are known to be made along with the target mRNA.

### Instability of the target mRNA and protein

One might expect that instability of target mRNA might limit expression in some cases, although in each case that has been examined, substantial amounts of target mRNA seem to accumulate. Instability of certain target proteins might also be expected, although BL21 is deficient in the *lon* and *ompT* proteases and many proteins produced in this strain are quite stable. Some relatively short proteins produced by out-of-frame fusions are also quite stable in this strain, whereas others are so rapidly degraded they remain undetected by pulse labeling.

## IX. Acknowledgements

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# X.

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## XII. Academic & Non-profit Laboratory Assurance Letter

The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is the subject of patent applications assigned to Brookhaven Science Associates (BSA). This technology, including bacteria, phages and plasmids that carry the gene for T7 RNA polymerase, is made available on the following conditions:

1. The T7 expression system is to be used for noncommercial research purposes only. A license is required for any commercial use, including use of the T7 system for research purposes or for production purposes by any commercial entity. Information about commercial licenses may be obtained from the Patent Office, Brookhaven National Laboratory, Upton, New York, 11973, Telephone: (631) 344-7134. Contact: Christine Brakel.
2. No materials that contain the cloned gene for T7 RNA polymerase may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this assurance letter and agrees to be bound by its terms. This limitation applies to any of the following materials that are included in this kit and to any derivatives you may make of them:

<i>E. coli</i> B834(DE3)	<i>E. coli</i> Origami 2(DE3)	<i>E. coli</i> Rosetta-gami™(DE3)
<i>E. coli</i> B834(DE3)pLysS	<i>E. coli</i> Origami 2(DE3)pLysS	<i>E. coli</i> Rosetta-gami(DE3)pLysS
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> Origami 2(DE3)pLacI	<i>E. coli</i> Rosetta-gami(DE3)pLacI
<i>E. coli</i> BL21(DE3)pLysS	<i>E. coli</i> Origami B(DE3)	<i>E. coli</i> Rosetta-gami 2(DE3)
<i>E. coli</i> BL21(DE3)pLysE	<i>E. coli</i> Origami B(DE3)pLysS	<i>E. coli</i> Rosetta-gami 2(DE3)pLysS
<i>E. coli</i> BL26(DE3)pLysE	<i>E. coli</i> Origami B(DE3)pLacI	<i>E. coli</i> Rosetta-gami 2(DE3)pLacI
<i>E. coli</i> BLR(DE3)	<i>E. coli</i> Rosetta™(DE3)	<i>E. coli</i> Rosetta-gami B(DE3)
<i>E. coli</i> BLR(DE3)pLysS	<i>E. coli</i> Rosetta(DE3)pLysS	<i>E. coli</i> Rosetta-gami B(DE3)pLysS
<i>E. coli</i> HMS174(DE3)	<i>E. coli</i> Rosetta(DE3)pLacI	<i>E. coli</i> Rosetta-gami B(DE3)pLacI
<i>E. coli</i> HMS174(DE3)pLysS	<i>E. coli</i> Rosetta 2(DE3)	<i>E. coli</i> Tuner™(DE3)
<i>E. coli</i> HMS174(DE3)pLysE	<i>E. coli</i> Rosetta 2(DE3)pLysS	<i>E. coli</i> Tuner(DE3)pLysS
<i>E. coli</i> NovaBlue(DE3)	<i>E. coli</i> Rosetta 2(DE3)pLacI	<i>E. coli</i> Tuner(DE3)pLacI
<i>E. coli</i> Origami™(DE3)	<i>E. coli</i> RosettaBlue™(DE3)	Bacteriophage λCE6
<i>E. coli</i> Origami(DE3)pLysS	<i>E. coli</i> RosettaBlue(DE3)pLysS	Bacteriophage λDE3
<i>E. coli</i> Origami(DE3)pLacI	<i>E. coli</i> RosettaBlue(DE3)pLacI	

3. The initial purchaser may refuse to accept the above conditions by returning the kit unopened and the enclosed materials unused. By accepting or using the kit or the enclosed materials, you agree to be bound by the foregoing conditions.

## XIII. Bacterial Strain Non- Distribution Agreement

By purchase of the Origami™, Origami 2, Origami B, Rosetta™, Rosetta 2, RosettaBlue™, Rosetta-gami™, Rosetta-gami 2, or Rosetta-gami B host strains and acceptance of the following terms, Novagen grants a limited license to use the Origami, Origami 2, Origami B, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B host strains for the cloning and expression of genes. The intent of this license is not to limit the research use of these materials, but to protect against unauthorized commercial distribution of the strains by third parties.

1. The Origami, Origami 2, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B host strains or any derivative therefrom is not to be offered for resale or distributed outside your laboratory.
2. Gene clones and libraries in the Origami, Origami 2, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B host strains may be distributed for research purposes only, provided that the recipient acknowledge the foregoing condition.
3.  $\lambda$ DE3 lysogens of host strains Origami, Origami 2, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B are covered by U.S. Patent No. 4,952,496. Commercial customers must obtain a research license agreement from Brookhaven Science Associates before purchase.

The initial purchaser may refuse to accept the above conditions by returning the kit unopened and the enclosed materials unused. By accepting or using the kit or the enclosed materials, you agree to be bound by the foregoing conditions.

# XIV.

## Appendix:

### pET System Related Products

## A. pET System Host Strains and Lambda Phage

### $\lambda$ DE3 lysogens for protein expression

The pET expression hosts are lysogens of bacteriophage  $\lambda$ DE3, as indicated by the (DE3) in their names and are suitable for production of protein from target genes cloned in pET vectors. The pLysS and pLysE designation is given to hosts carrying a pET-compatible plasmid that encodes T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase. These strains are used to control basal expression of T7 RNA polymerase prior to induction and thus stabilize pET recombinants encoding target proteins that affect cell growth and viability.

Strain	Size	Competent Cells Cat. No.	Glycerol Stock Cat. No.	Application
B834(DE3)	0.4 ml 1.0 ml	69041-3 69041-4		Selenomethionine and <sup>35</sup> S-met labeling
B834(DE3)pLysS	0.4 ml 1.0 ml	69042-3 69042-4		
BL21(DE3)	0.2 ml 0.4 ml 1.0 ml	69450-3 69450-4	69387-3	Protects target protein from <i>lon</i> and <i>ompT</i> proteases
HT96™ BL21(DE3)	1 plate 4 plates	71012-3 71012-4		
BL21(DE3) Singles™	11 rxn 22 rxn	70235-3 70235-4		
BL21(DE3)pLysS	0.2 ml 0.4 ml 1.0 ml	69451-3 69451-4	69388-3	
BL21(DE3)pLysS Singles	11 rxn 22 rxn	70236-3 70236-4		
BL21(DE3)pLysE	0.2 ml		69389-3	
BLR(DE3)	0.4 ml 1.0 ml	69053-3 69053-4		A <i>recA</i> <sup>-</sup> derivative of BL21 that may stabilize target plasmids containing repetitive sequences.
BLR(DE3)pLysS	0.2 ml 0.4 ml 1.0 ml	69956-3 69956-4	69209-3	
HMS174(DE3)	0.4 ml 1.0 ml	69453-3 69453-4		A K-12 <i>recA</i> <sup>-</sup> strain that may stabilize target plasmids containing repetitive sequences
HMS174(DE3)pLysS	0.4 ml 1.0 ml	69454-3 69454-4		
HMS174(DE3)pLysE	0.2 ml		69393-3	
NovaBlue(DE3)	0.4 ml 1.0 ml	69284-3 69284-4		High stringency host with <i>recA</i> , <i>endA1</i> , and <i>lacI<sup>H</sup></i> mutations. Recommended for use with the NovaTope™ System
Origami™(DE3)	0.2 ml 0.4 ml 1.0 ml	70627-3 70627-4	70617-3	Allows disulfide bond formation in <i>E. coli</i> cytoplasm
Origami(DE3) Singles	11 rxn 22 rxn	70630-3 70630-4		
Origami(DE3)pLysS	0.2 ml 0.4 ml 1.0 ml	70628-3 70628-4	70618-3	
Origami(DE3)pLysS Singles	11 rxn 22 rxn	70631-3 70631-4		

Strain	Size	Competent Cells Cat. No.	Glycerol Stock Cat. No.	Application
Origami™ 2(DE3)	0.2 ml		71348-3	Allows disulfide bond formation in <i>E. coli</i> cytoplasm and is kanamycin sensitive.
	0.4 ml	71345-3		
	1.0 ml	71345-4		
Origami 2(DE3) Singles™	11 rxn	71408-3		
	22 rxn	71408-4		
Origami™ 2(DE3)pLysS	0.2 ml		71349-3	
	0.4 ml	71346-3		
	1.0 ml	71346-4		
Origami 2(DE3)pLysS Singles	11 rxn	71409-3		
	22 rxn	71409-4		
Origami B(DE3)	0.4 ml	70837-3		Allows disulfide bond formation in <i>E. coli</i> cytoplasm and protects target protein from <i>lon</i> and <i>ompT</i> proteases. A derivative of Tuner™ ( <i>lacY1</i> ) enabling low levels expression in all cells for solubility and activity enhancement.
	1.0 ml	70837-4		
Origami B(DE3)pLysS	0.4 ml	70839-3		
	1.0 ml	70839-4		
Rosetta™ (DE3)	0.4 ml	70954-3		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA).
	1.0 ml	70954-4		
Rosetta(DE3)pLysS	0.4 ml	70956-3		
	1.0 ml	70956-4		
Rosetta 2(DE3)	0.4 ml	71397-3		Enhances expression of proteins that contain seven codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CAU, CCC, GGA, CGG).
	1.0 ml	71397-4		
Rosetta 2(DE3) Singles	11 rxn	71400-3		
	22 rxn	71400-4		
Rosetta 2(DE3)pLysS	0.4 ml	71403-3		
	1.0 ml	71403-4		
Rosetta 2(DE3)pLysS Singles	11 rxn	71401-3		
	22 rxn	71401-4		
RosettaBlue™ (DE3)	0.4 ml	71059-3		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA) and is a high stringency host with <i>recA</i> , <i>endA</i> and <i>lac<sup>H</sup></i> mutations.
	1.0 ml	71059-4		
RosettaBlue(DE3)pLysS	0.4 ml	71034-3		
	1.0 ml	71034-4		
Rosetta-gami™ (DE3)	0.4 ml	71055-3		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA). Allows disulfide bond formation in the <i>E. coli</i> cytoplasm.
	1.0 ml	71055-4		
Rosetta-gami(DE3)pLysS	0.4 ml	71057-3		
	1.0 ml	71057-4		
Rosetta-gami 2(DE3)	0.4 ml	71351-3		Enhances expression of proteins that contain seven codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CAU, CCC, GGA, CGG). Allows disulfide bond formation in the <i>E. coli</i> cytoplasm. Kanamycin sensitive.
	1.0 ml	71351-4		
Rosetta-gami 2(DE3)pLysS	0.4 ml	71352-3		
	1.0 ml	71352-4		
Rosetta-gami B(DE3)	0.4 ml	71136-3		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA). Allows disulfide bond formation in the <i>E. coli</i> cytoplasm. A derivative of Tuner™ ( <i>lacY1</i> ) enabling low levels expression in all cells for solubility and activity enhancement.
	1.0 ml	71136-4		
Rosetta-gami B(DE3)pLysS	0.4 ml	71137-3		
	1.0 ml	71137-4		
Tuner™ (DE3)	0.4 ml	70623-3		Enables adjustable levels of expression in all cells due to the <i>lacY1</i> mutation; lower IPTG levels may enhance solubility and activity of the target protein.
	1.0 ml	70623-4		
Tuner(DE3)pLysS	0.4 ml	70624-3		
	1.0 ml	70624-4		

### Isogenic host strains for initial cloning, controls, and expression

These host strains are isogenic with the  $\lambda$ DE3 lysogens used for protein expression but lack a source of T7 RNA polymerase. These strains can be used for T7-based expression by infection with  $\lambda$ CE6, or for expression from *E. coli* promoters. Only the NovaBlue, NovaXG, and NovaXGF' strains are recommended for initial cloning.

Strain	Size	Competent Cells Cat. No.	Glycerol Stock Cat. No.	Application
B834	0.2 ml		69287-3	Selenomethionine and <sup>35</sup> S-met labeling
BL21	0.2 ml 0.4 ml 1.0 ml	69449-3 69449-4	69386-3	Protects target protein from <i>lon</i> and <i>ompT</i> proteases.
BLR	0.2 ml 0.4 ml 1.0 ml	69052-3 69052-4	69207-3	A <i>recA</i> <sup>-</sup> derivative of BL21 that may stabilize target plasmids containing repetitive sequences.
HMS174	0.4 ml 1.0 ml	69452-3 69452-4		A <i>recA</i> <sup>-</sup> K-12 strain that may stabilize target plasmids containing repetitive sequences.
NovaBlue	0.2 ml 0.4 ml 1.0 ml	69825-3 69825-4	69009-3	High stringency host with the <i>recA</i> <sup>-</sup> <i>endA</i> <sup>-</sup> and <i>lacI</i> <sup>H</sup> mutations.
HT96™ NovaBlue	1 plate 4 plates	71011-3 71011-4		
NovaBlue Singles™	11 rxn 22 rxn	71081-3 71081-4		
NovaBlue GigaSingles™	11 rxn 22 rxn	71227-3 71227-4		
NovaBlue T1 <sup>h</sup> Singles	11 rxn 22 rxn	71318-3 71318-4		High stringency host with the <i>recA</i> <sup>-</sup> <i>endA</i> <sup>-</sup> and <i>lacI</i> <sup>H</sup> mutations. T1 and T5 phage resistant.
NovaXG Zappers	10 rxn 20 rxn	71315-3 71315-4		Methylation restriction minus. T1 and T5 phage resistant.
NovaXGF' Zappers	10 rxn 20 rxn	71317-3 71317-4		Methylation restriction minus. T1 and T5 phage resistant.
Origami™	0.4 ml 1.0 ml	70626-3 70626-4		Allows disulfide bond formation in <i>E. coli</i> cytoplasm.
Origami 2	0.4 ml 1.0 ml	71344-3 71344-4		Allows disulfide bond formation in <i>E. coli</i> cytoplasm. Kanamycin sensitive.
Origami B	0.4 ml 1.0 ml	70836-3 70836-4		Allows disulfide bond formation in <i>E. coli</i> cytoplasm. A derivative of Tuner™ ( <i>lacY1</i> ) enabling low levels expression in all cells for solubility and activity enhancement.
Rosetta™	0.4 ml 1.0 ml	70953-3 70953-4		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA).
Rosetta 2	0.4 ml 1.0 ml	71402-3 71402-4		Enhances expression of proteins that contain seven codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA, CGG).
RosettaBlue™	0.4 ml 1.0 ml	71058-3 71058-4		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA) and is a high stringency host with <i>recA</i> <sup>-</sup> , <i>endA</i> <sup>-</sup> and <i>lacI</i> <sup>H</sup> mutations.
Rosetta-gami™	0.4 ml 1.0 ml	71054-3 71054-4		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA). Allows disulfide bond formation in the <i>E. coli</i> cytoplasm.
Rosetta-gami 2	0.4 ml 1.0 ml	71350-3 71350-4		Enhances expression of proteins that contain seven codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA, CGG). Allows disulfide bond formation in the <i>E. coli</i> cytoplasm. Kanamycin sensitive.
Rosetta-gami B	0.4 ml 1.0 ml	71135-3 71135-4		Enhances expression of proteins that contain six codons rarely used in <i>E. coli</i> (AGG, AGA, AUA, CUA, CCC, GGA). Allows disulfide bond formation in the <i>E. coli</i> cytoplasm. A derivative of Tuner™ ( <i>lacY1</i> ) enabling low levels expression in all cells for solubility and activity enhancement.
Tuner™	0.4 ml 1.0 ml	70622-3 70622-4		Enables adjustable levels of expression in all cells due to the <i>lacY1</i> mutation; lower levels may enhance solubility and activity of the target protein.

## pET host strain competent cell sets

The competent cell sets easily facilitate optimization of vector/host strain combinations.

Competent Cell Set	Size	Cat. No.	Strains
Non-λDE3 Competent Cell Set 1	0.2 ml each	71211-3	BL21, NovaBlue, Origami™, Rosetta™ 2, Rosetta-gami™ B
(DE3) Competent Cell Set 1	0.2 ml each	71207-3	BL21(DE3), BLR(DE3), HMS174(DE3), NovaBlue(DE3), Tuner™(DE3)
(DE3) Competent Cell Set 2	0.2 ml each	71208-3	Origami(DE3), Origami B(DE3), Rosetta 2(DE3), RosettaBlue™, Rosetta-gami(DE3), Rosetta-gami B(DE3)
(DE3)pLysS Competent Cell Set 1	0.2 ml each	71209-3	BL21(DE3)pLysS, BLR(DE3)pLysS, HMS174(DE3)pLysS, Tuner(DE3)pLysS
(DE3)pLysS Competent Cell Set 2	0.2 ml each	71210-3	Origami(DE3)pLysS, Origami B(DE3)pLysS, Rosetta 2(DE3)pLysS, RosettaBlue(DE3)pLysS, Rosetta-gami(DE3)pLysS, Rosetta-gami B(DE3)pLysS
BL21 Competent Cell Set	2 × 0.2 ml each	70232-3	BL21, BL21(DE3), BL21(DE3)pLysS
BLR Competent Cell Set	2 × 0.2 ml each	70233-3	BLR, BLR(DE3), BLR(DE3)pLysS
HMS174 Competent Cell Set	2 × 0.2 ml each	70234-3	HMS174, HMS174(DE3), HMS174(DE3)pLysS
Origami Competent Cell Set	2 × 0.2 ml each	70670-3	Origami, Origami(DE3), Origami(DE3)pLysS
Origami 2 Competent Cell Set	2 × 0.2 ml each	71431-3	Origami 2, Origami 2(DE3), Origami 2(DE3)pLysS
Origami B Competent Cell Set	2 × 0.2 ml each	70911-3	Origami B, Origami B;(DE3), Origami B(DE3)pLysS
Rosetta 2 Competent Cell Set	2 × 0.2 ml each	71405-3	Rosetta 2, Rosetta 2(DE3), Rosetta 2(DE3)pLysS
RosettaBlue Competent Cell Set	2 × 0.2 ml each	71079-3	RosettaBlue, RosettaBlue(DE3), RosettaBlue(DE3)pLysS
Rosetta-gami Competent Cell Set	2 × 0.2 ml each	71080-3	Rosetta-gami, Rosetta-gami(DE3), Rosetta-gami(DE3)pLysS
Rosetta-gami 2 Competent Cell Set	2 × 0.2 ml each	71432-3	Rosetta-gami 2, Rosetta-gami 2(DE3), Rosetta-gami 2(DE3)pLysS
Tuner Competent Cell Set	2 × 0.2 ml each	70726-3	Tuner, Tuner(DE3), Tuner(DE3)pLysS

## pET System lambda phage

Lambda Phage	Size	Cat. No.	Application
Bacteriophage CE6	0.2 ml 10 ml	69390-3 69390-4	A recombinant phage used to provide a source of T7 RNA Polymerase to susceptible host cells carrying pET plasmids.
λDE3 Lysogenization Kit	10 rxn	69734-3	Designed for integration of DE3 prophage into <i>E. coli</i> host cell chromosome such that the lysogenized host can be used to express target genes cloned in pET vectors.
λDE3 Lysogenization Kit plus pLysS & pLysE	10 rxn	69725-3	

## B. Detection/Assay Tools for Fusion Tags

The identity and quantity of the target protein can be determined by Western blotting and quantitation assays with target protein-specific antibodies, conjugates or assays based on pET vector-encoded fusion partners. Specific protocols for Western blotting and rapid assays using detection reagents and kits are available at [www.novagen.com](http://www.novagen.com) and listed in the following table.

For size estimation during Western blotting, load Perfect Protein™ Western Blot Markers (Cat. No. 69959-3) or Trail Mix™ Western Markers (71047-3, 71048-3) in a lane adjacent to the unknown sample. Both sets of markers carry the S•Tag™ and His•Tag® sequences so they can be detected using a S-protein conjugate (McCormick et al., 1994) or the His•Tag Monoclonal Antibody (Fourrier et al., 2001).

**Note:** *Detection of Trail Mix Western Markers with the His•Tag Monoclonal Antibody and Goat Anti-Mouse IgG HRP Conjugate (H+L) is not recommended. As an alternative, use Perfect Protein Western Markers.*

Western Blotting	Cat. No.	Size	User Protocol No./Application
<b>GST•Tag™ detection</b>			
GST•Tag Monoclonal Antibody	71097-3	50 µg 250 µg	TB325 IF, IP, QA, WB
GST•Tag Antibody Plate	71347-3 71347-4	1 plate 5 plates	TB412 ELISA
<b>His•Tag® detection</b>			
His•Tag Monoclonal Antibody	70796-4 70796-3	3 µg 100 µg	TB283 IF, IP, WB
His•Tag AP Western Reagents	70972-3	25 blots	TB283 colorimetric detection
His•Tag AP LumiBlot™ Reagents	70973-3	25 blots	TB283 chemiluminescent detection
His•Tag HRP LumiBlot Reagents	70974-3	25 blots	TB283 chemiluminescent detection
His•Tag Antibody Plate	71184-3 71184-4	1 plate 5 plates	TB345 ELISA
<b>HSV•Tag® detection</b>			
HSV•Tag Monoclonal Antibody	69171-3 69171-4	40 µg 200 µg	TB067 WB
<b>Nus•Tag™ detection</b>			
Nus•Tag Monoclonal Antibody	71127-3 71127-4	50 µg 250 µg	TB328 WB
<b>S•Tag™ II detection</b>			
S-protein AP Conjugate	69598-3	50 µl	TB097 WB
S-protein HRP Conjugate	69047-3	50 µl	TB136 WB
Biotinylated S-protein	69218-3	250 µl	WB
S-protein FITC Conjugate	69060-3	200 µl	TB143 IF
S•Tag AP Western Blot Kit	69213-3	25 blots	TB082 colorimetric detection
S•Tag AP LumiBlot Kit	69099-3	25 blots	TB164 chemiluminescent detection
S•Tag HRP LumiBlot Kit	69058-3	25 blots	TB145 chemiluminescent detection
<b>Strep•Tag® II detection</b>			
Strep•Tag II Monoclonal Antibody	71590-3	100 µg	TB445 WB
Strep•Tag II Antibody HRP Conjugate	71591-3	75 µl	TB446 WB

Western Blotting	Cat. No.	Size	User Protocol No./Application
<b>T7•Tag® detection</b>			
T7•Tag Monoclonal Antibody	69522-3 69522-4	50 µg 250 µg	TB015 IF, IP, WB
Biotinylated T7•Tag Antibody	69968-3	125 µl	TB106 WB
T7•Tag Antibody HRP Conjugate	69048-3	100 µl	TB137 WB
T7•Tag Antibody AP Conjugate	69999-3	50 µl	TB112 WB
T7•Tag AP LumiBlot Kit	70237-3	25 blots	TB212 chemiluminescent detection
T7•Tag HRP LumiBlot Kit	70238-3	25 blots	TB213 chemiluminescent detection
Western Blot Protein Markers	Cat. No.	Size	User Protocol No./Size Standards
Perfect Protein™ Western Markers	69959-3	25 lanes	TB102; 15, 25, 35, 50, 75, 100 and 150 kDa
Trail Mix™ Western Markers	70982-3	25 lanes	TB310; 15, 25, 35, 50, 75, 100 and 150 kDa, and 15, 16, 100 kDa prestained markers
Quantitative Assay	Cat. No.	Size	User Protocol No./Sensitivity
FRETWorks™ S•Tag™ Assay Kit	70724-3 70724-4	100 assays 1000 assays	TB251; fluorescent assay, Limit < 1 fmol
S•Tag Rapid Assay Kit	69212-3	100 assays	TB082; Limit 20 fmol
GST•Tag™ Assay Kit	70532-3	100 assays	TB236; colorimetric assay, Limit 8 pmol

IF: immunofluorescence, IP: immunoprecipitation, QA: quantitative assay, WB: Western blotting

## C. Purification Tools

A brief description of products for extract preparation and affinity chromatography is indicated below. For detailed information, see the indicated User Protocol available at [www.novagen.com](http://www.novagen.com)

Extraction Reagents	Cat. No.	Size	User Protocol No./Capacity and Features
BugBuster® Protein Extraction Reagent	70584-3 70584-4	100 ml 500 ml	TB245 Use 5 ml/g wet cell paste. Tris-buffered.
BugBuster Master Mix	71456-3 71456-4	100 ml 500 ml	TB245 Use 5 ml/g wet cell paste. Tris buffered and premixed with Benzonase® Nuclease and rLysozyme™ Solution.
BugBuster HT Protein Extraction Reagent	70922-3 70922-4	100 ml 500 ml	TB245 Use 5 ml/g wet cell paste. Tris-buffered and premixed with Benzonase Nuclease.
BugBuster 10X Protein Extraction Reagent	70921-3 70921-4 70921-5	10 ml 50 ml 100 ml	TB245 Dilute to 1X with choice of buffer and use 5 ml/g wet cell paste.
BugBuster (primary amine-free) Extraction Reagent	70923-3 70923-4	100 ml 500 ml	TB245 Use 5 ml/g wet cell paste. PIPPS-buffered.
PopCulture® Reagent	71092-3 71092-4 71092-5	15 ml 75 ml 250 ml	TB323 Use 0.1 volume per ml of culture.
rLysozyme™ Solution	71110-3 71110-4 71110-5	300 KU 1200 KU 6000 KU	TB334 and TB323 Use 40 U per 1 ml of culture volume with PopCulture Reagent and 1 KU per 1 ml BugBuster Reagent.
Benzonase Nuclease, Purity > 90%	70746-4 70746-3	2.5 KU 10 KU	TB245, 323, 261 Use 25 U per ml original culture volume with PopCulture and BugBuster Reagent.
Benzonase Nuclease HC, Purify >90%	71205-3	25 KU	TB245, 323, 261 Use 25 U per ml original culture volume with PopCulture and BugBuster Reagent.

Extraction Reagents	Cat. No.	Size	User Protocol No./Capacity and Features
Lyonase™ Bioprocessing Reagent	71230-3	0.2 ml	TB245, 323, 334 Use 10 µl per gram cell paste with BugBuster® and 2 µl per ml original culture volume with PopCulture® reagent.
	71230-4	1 ml	
	71230-5	5 × 1 ml	
BugBuster Plus Benzonase® Kit	70750-3		TB245 Sufficient for protein extraction from 20 g cell paste.
BugBuster Plus Lyonase Kit	71370-3 71370-4		TB245 Two kit sizes provide sufficient reagents for protein extraction from either 20 or 100 g cell paste.
GST•Tag™ purification	Cat. No.	Size	User Protocol No./Capacity and Features
GST•Bind™ Resin	70541-3	10 ml	TB235 Capacity is 5–8 mg/ml settled resin.
	70541-4	50 ml	
GST•Bind Buffer Kit	70534-3		TB235 All buffers for ten 2.5 ml columns.
GST•Mag™ Agarose Beads	71084-3	2 × 1 ml	TB235 Magnetic agarose beads. Capacity up to 2 mg/ml settled volume.
	71084-4	10 × 1 ml	
BugBuster GST•Bind Purification Kit	70794-3		TB235 GST•Bind Resin and Buffer, BugBuster, Benzonase and Chromatography Columns.
PopCulture GST•Mag Purification Kit	71113-3		TB235 Process 40 × 3 ml cultures purifying up to 150 µg per 3 ml culture.
RoboPop™ GST•Mag Purification Kit	71102-3		TB327 Purify up to 4.8 mg per 96 wells.
RoboPop GST•Bind Purification Kit	71189-3		TB346 Purify up to 76.8 mg per 96 wells.
His•Tag® purification	Cat	Size	User Protocol No./Capacity and Features
Ni-NTA His•Bind® Resin	70666-3	10 ml	TB273 Capacity is 5–10 mg/ml settled resin
	70666-4	25 ml	
	70666-5	100 ml	
Ni-NTA Superflow™	70691-3	10 ml	TB273 Capacity is 5–10 mg/ml settled resin, high flow rates and pressures.
	70691-4	25 ml	
	70691-5	100 ml	
Ni-NTA Buffer Kit	70889-3		TB273 All buffers for native purification using Ni-NTA His•Bind and Ni-NTA Superflow resins.
His•Bind® Resin	69670-3	10 ml	TB054 Capacity is 8 mg/ml settled resin.
	69670-4	50 ml	
	69670-5	100 ml	
His•Bind Buffer Kit	69755-3		TB054 All buffers for native purification using His•Bind Resin.
His•Bind Columns	70971-3	pkg/5	TB054 pre-packed, pre-charged; Capacity is 10 mg per column.
	70971-4	pkg/25	
His•Bind Quick Columns	70159-3	pkg/12	TB054 pre-packed, pre-charged, requires vacuum, Capacity is 5 mg per column.
	70159-4	pkg/60	
His•Bind Quick 300 Cartridges	70155-3	pkg/10	TB054 pre-packed, pre-charged, Capacity is 0.5 mg per cartridge
	70155-4	pkg/50	
His•Bind Quick 900 Cartridges	70153-3	pkg/10	TB054 pre-packed, pre-charged, Capacity is 2 mg per cartridge
	70153-4	pkg/50	
His•Mag™ Agarose Beads	71002-3 71002-4	2 ml 10 ml	TB054 magnetic agarose beads, pre-charged, Capacity is 5 mg per ml settled beads.
His•Bind Quick Buffer Kit	70665-3		TB054 all buffers for native purification using His•Bind Columns, Quick Columns, Cartridges and His•Mag Agarose Beads. No charge buffer included.
His•Bind Purification Kit	70239-3		TB054 10 ml His•Bind Resin, Buffers and Chromatography Columns
BugBuster® Ni-NTA His•Bind Purification Kit	70751-3		TB273 10 ml Ni-NTA His•Bind Resin, BugBuster, Benzonase and Chromatography Columns.
BugBuster His•Bind Purification Kit	70793-3		TB054 10 ml His•Bind Resin and Buffer, BugBuster, Benzonase and Chromatography Columns.
PopCulture® His•Mag™ Purification Kit	71114-3		TB054 Process 40 × 3 ml cultures purifying up to 375 µg per 3 ml culture.
RoboPop™ His•Mag Purification Kit	71103-3		TB327 Purify up to 12 mg per 96 wells.
RoboPop Ni-NTA His•Bind Purification Kit	71257-3		TB368 Purify up to 38.4 mg per 96 wells.
S•Tag™ purification	Cat. No.	Size	User Protocol No./Capacity and Features
S-protein Agarose	69704-3	2 ml	TB087, TB160 Purify up to 1 mg per 2 ml settled resin
	69704-4	5 × 2 ml	
S•Tag Thrombin Purification Kit	69232-3		TB087 Purify and cleave up to 1 mg target protein per kit (2 ml settled resin)

S•Tag Purification	Cat. No	Size	User Protocol No./Capacity and Features
S•Tag rEK Purification Kit	69065-3		TB160 Purify and cleave up to 1 mg target protein per kit (2 ml settled resin)
Strep•Tag® purification	Cat. No.	Size	User Protocol No./Capacity and Features
Strep•Tactin® Superflow™ Agarose	71592-3 71592-4	2 ml 10 ml	TB449 Capacity is up to 3 mg/ml settled resin, high flow rates and pressures
Strep•Tactin Superflow Column, 0.2 ml	71594-3	5 columns	TB449 Capacity is up to 0.6 mg per column, suitable for gravity flow chromatography
Strep•Tactin Superflow Column, 1 ml	71593-3	5 columns	TB449 Capacity is up to 3 mg per column, suitable for gravity flow chromatography
Strep•Tactin Superflow Cartridge, 1 ml	71595-3	5 cartridges	TB449 Capacity is up to 3 mg per column, suitable for FPLC/HPLC, can be used with syringes
Strep•Tactin Superflow Cartridge, 5 ml	71596-3 71596-4	1 cartridge 5 cartridges	TB449 Capacity is up to 15 mg per column, suitable for FPLC/HPLC, can be used with syringes
Strep•Tactin MacroPrep® Resin	71597-3 71597-4	2 ml 10 ml	TB449 Capacity is up to 3 mg/ml settled resin,
Strep•Tactin MacroPrep Cartridge	71598-3	5 columns	TB449 Capacity is up to 3 mg per cartridge, suitable for FPLC/HPLC, can be used with syringes
Strep•Tactin Buffer Kit	71613-3		TB449 All buffers for purification
Strep•Tactin HT96™ Purification Kit	71605-3	1 plate	TB447 Capacity is up to 100 µg per well
Strep•Tactin SpinPrep™ Kit	71608-3	25 columns	TB449 Capacity is up to 150 µg per column
T7•Tag Antibody Agarose	69026-3	2 × 1 ml	TB125 minimum 300 µg T7•Tag β-galactosidase per ml settled resin. May vary between target proteins.
T7•Tag Affinity Purification Kit	69025-3		TB125 minimum 300 µg T7•Tag β-galactosidase per ml settled resin. May vary between target proteins. One ml of agarose, buffers and columns for purification.
Proteases and Cleavage Capture Kits	Cat. No.	Size	User Protocol No./Description
Thrombin, Restriction Grade	69671-3	50 U	TB188
Biotinylated Thrombin	69672-3	50 U	TB188
Thrombin Cleavage Capture Kit	69022-3		TB188 50 U Biotinylated Thrombin and Streptavidin Agarose for Biotinylated Thrombin removal
Recombinant Enterokinase	69066-3	50 U	TB150
Enterokinase Cleavage Capture Kit	69067-3		TB150 50 U Recombinant Enterokinase and Ekapture™ Agarose for enterokinase removal
Factor Xa, Restriction Grade	69036-3	400 U	TB205
Factor Xa Cleavage Kit	69037-3		TB205 400 U Factor Xa and Xarrest™ Agarose for Factor Xa removal
HRV 3C Protease	71493-3	500 U	TB420