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Technical Service: 800 207-0144

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 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. 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 (see Section I.G. for details). Once established in a non-expression host, plasmids are then transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control, and expression is induced by the addition of IPTG. Two types of T7 promoter 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.

To get started quickly, move ahead to page 15.

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 system provides the core reagents needed. The background information following *System Components* 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 the last page of this manual or in the letter received with your kit.

C. System Components

pET Expression Systems provide 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[1,2]
- Induction control clone, glycerol stock
- Novagen Vector Diskette containing all vector sequences (compatible with Macintosh and DOS/Windows)

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 contain a set of three host strains ready for high-efficiency transformation of pET recombinants. These kits include the following additional components, which are sufficient for up to 10 transformations in each host:

- One 0.2 ml aliquot of each host NovaBlue, BL21(DE3) and BL21(DE3)pLysS as pretested competent cells
- SOC medium
- Test Plasmid

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

[2] The pET Trx Fusion System 32 includes AD494 series hosts strains in addition to the BL21 series hosts.

Components available separately:

Product	Size	Cat. No.
pET Vector DNA		*
pET Host Strains & Competent Cells		see p. 11
Novagen Vector Diskette		69447

* Please refer to the Novagen catalog or www.novagen.com for a complete list.

D. The pET Vectors

The pET vectors were originally constructed by Studier and colleagues (Studier and Moffatt, 1986; Rosenberg et al., 1987; Studier et al., 1990). The newer pET derivatives developed at Novagen were designed with enhanced features to permit easier subcloning, detection, and purification of target proteins. Two general categories of vectors are available: transcription and translation vectors.

- Transcription vectors are designed for expression of target genes that already carry their own prokaryotic ribosome binding site and ATG start codon.
- Translation vectors contain the highly efficient ribosome binding site from the phage T7 major capsid protein.

  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 ATG start codon.

Vector Characteristics and Cloning Strategy

In general, translation vectors are used for the expression of target genes derived from eukaryotic sources, and transcription vectors are used for target genes derived from prokaryotic genes (which usually carry compatible ribosome binding sites).

Many strategies can be used for subcloning a protein-coding region of DNA into a pET vector for expression. The most convenient strategy is to use the unique restriction sites found in the multiple cloning region of the pET vectors for cloning inserts in a defined orientation using two different sites.

Ligation-Independent Cloning (LIC) of PCR Products

Another convenient method that does not require restriction digestion is the ligation-independent cloning (LIC) method. Ligation-independent cloning was developed for the directional cloning of PCR products without restriction enzyme digestion or ligation reactions (Aslanidis and de Jong, 1990; Haun et al., 1992). LIC vectors are created by treating a linearized backbone with T4 DNA polymerase in the presence of only one dNTP. The 3'→5' exonuclease activity of T4 DNA polymerase removes nucleotides until it encounters a residue corresponding to the single dNTP present in the reaction mix. At this point the 5'→3' polymerase activity of the enzyme counteracts the exonuclease activity to effectively prevent further excision. Plasmid sequences adjacent to the site of linearization are typically designed to produce specific non-complementary 12 to 14 base single stranded overhangs in the LIC vector. Compatible inserts with complementary overhangs are generated by building appropriate 5' extensions into the primers. The PCR product is purified to remove dNTPs (and original plasmid if it was used as template) and then treated with T4 DNA polymerase in the presence of the appropriate dNTP to generate the specific vector-compatible overhangs. Cloning is very fast and efficient because only the desired product is formed by annealing. The annealed LIC vector and insert are transformed into competent *E. coli* cells. Covalent bond formation at the vector-insert junctions occurs within the cell to yield circular plasmid.

Novagen offers LIC vectors as linearized, LIC-modified DNA in which the LIC overhang encodes an enterokinase or Factor Xa cleavage site. The vectors are designed to enable the removal of all vector-encoded sequence from the expressed fusion protein upon cleavage with these proteases. Kits include LIC Vector, annealing reagents and competent cells (NovaBlue Singles™, BL21(DE3) and BL21(DE3)pLysS).

pET LIC Vector Kits	Size	Cat. No.
pET Ek/LIC Vector Kits		
pET-30 Ek/LIC Vector Kit	20 rxn	69077-3
pET-32 Ek/LIC Vector Kit	20 rxn	69076-3
pET-34 Ek/LIC Vector Kit	20 rxn	70114-3
pET-36 Ek/LIC Vector Kit	20 rxn	70145-3
pET Ek/LIC Combo Kit	40 rxn	70255-3
pET Xa/LIC Vector Kits		
pET-30 Xa/LIC Vector Kit	20 rxn	70073-3
pET-32 Xa/LIC Vector Kit	20 rxn	70072-3
pET-35 Xa/LIC Vector Kit	20 rxn	70115-3
pET-37 Xa/LIC Vector Kit	20 rxn	70153-3

Fusion Tags

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, proteins without vector-encoded C-terminal fusions are obtained by including a translation stop codon in the insert.

However, various pET vectors also contain different sequences adjacent to the cloning sites that encode a number of peptide "tags", which perform various functions when fused with the target protein. Some of the fusion tags facilitate detection and purification of the target protein, whereas others increase the probability of biological activity by affecting solubility in the cytoplasm or export to the periplasm. The choice of cloning sites and strategies depends on the combination of tags desired, and the location of tags on the N-terminus, C-terminus, or both ends of the target protein. The various fusion tags and the vectors that carry them are summarized in the table on p. 6. 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.

Except for the pET-5 series, all of the pET 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 many pET plasmids contain the gene for ampicillin resistance (β-lactamase) in the same orientation as the target gene. If the T7 transcription terminator is removed during cloning, IPTG-dependent accumulation of β-lactamase (M_r 31,515) is usually observed along with the target protein, due to efficient read-through transcription by T7 RNA polymerase.

Fusion Tags Available for pET Constructs

Tag	N/C Terminal or Internal	Size (aa)	Basis for Detection and/or Purification	Applications	pET Vector Series
T7•Tag®	N, I	11 or 260	monoclonal antibody	western blot immunoprecipitation purification	3, 5, 9, 11, 17 17x, 21, 23, 24, 28, 33, pSCREEN
S•Tag™	N, I	15	S-protein (104aa) affinity	western blot quantitative assay purification	29, 30, 32, 34–37, 39–42, pSCREEN
His•Tag®	N, C, I	6, 8, or 10	metal chelation chromatography (native or denaturing)	His•Bind, resin purification	14–16, 19–42, pSCREEN
HSV•Tag®	C	11	monoclonal antibody	western blot immunofluorescence	25, 27
pelB/ompT	N	20/22	potential periplasmic localization	protein export/folding	12, 20, 22, 25, 26, 27
KSI	N	125	highly expressed hydrophobic domain	small protein/peptide production/purification	31
Trx•Tag™	N	109	thioredoxin	soluble protein, cyto-plasmic disulfide bond formation in *trxB*$^-$ hosts	32
PKA site	N	5	protein kinase A recognition site	*in vitro* phosphorylation	33
CBD_{clos}•Tag	N	156	polyclonal antibody, cellulose binding domain	western blot, purif./noncovalent immobilization	34, 35
CBD_{cenA}•Tag	N	114	polyclonal antibody, cellulose binding domain, periplasm/media	protein export, western blot, purif./noncovalent immobilization	36, 37
CBD_{cex}•Tag	C	107	polyclonal antibody, cellulose binding domain, periplasm/media	protein export, western blot, purif./noncovalent immobilization	38
Dsb•Tag™	N	208 (DsbA) 236 (DsbC)	potential periplasmic localization	soluble protein, peri-plasmic disulfide bond formation, isomerization	39, 40
GST•Tag™	N	220	glutathione affinity monoclonal antibody enzymatic activity	purification western blot quantitative assay	41, 42

E. Antibiotic Resistance

The selective markers amp (ampicillin resistance, also abbreviated Ap or *bla* for β-lactamase) and kan (kanamycin resistance) are available with the pET vectors and are indicated in the following table. Both types of selection have been widely used, but several simple guidelines are recommended when using vectors carrying the β-lactamase gene (see Section IV.C., *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 labs requiring GMP standards and when subcloning target genes from other ampicillin-resistant vectors. Ampicillin selection tends to be lost in cultures as the drug is degraded by the secreted β-lactamase enzyme and by the drop in pH that usually accompanies bacterial fermentation. Some ways to avoid this loss of drug resistance are to replace the medium with fresh ampicillin-containing media or to use the related drug, carbenicillin, which is less sensitive to low pH.

Another difference between kan^R and amp^R pET vectors involves the direction of transcription of the drug resistance gene. In amp^R pETs, the β-lactamase promoter is located downstream and in the same orientation as the T7 promoter. With the exception of the pET-5 series, all pET and pSCREEN™ plasmids have the native T7 transcription terminator (Tϕ) located before the β-lactamase promoter. However, this terminator is approximately 70% effective. Therefore, T7 RNA polymerase produces a small amount of β-lactamase RNA in addition to the target RNA, resulting in the accumulation of β-lactamase enzyme in induced cultures. In contrast, the kan

gene is in the opposite orientation from the T7 promoter, so there should not be an increase in kan gene product after induction due to read-through transcription from the T7 promoter.

F. pET Vector Characteristics

The following table lists the various cloning options available with the pET vectors. Note that 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. Cloning sites are shown on the maps provided with your pET vector kit.

Vector	amp^R	kan^R	T7	T7*lac*	f1 ori	His•Tag	T7•Tag^{11}	T7•Tag^{260}	S•Tag	Trx•Tag	CBD•Tag™	KSI	HSV•Tag	PKA	Dsb•Tag	GST•Tag	protease	signal seq.	LIC available
pET-3a-c	●		●				N												
pET-5a-c	●		●				N												
pET-9a-d		●	●				N												
pET-11a-d	●			●			N												
pET-12a-c	●		●															●	
pET-14b	●		●			N											T		
pET-15b	●			●		N											T		
pET-16b	●			●		N											X		
pET-17b	●		●				N												
pET-17xb	●		●					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							N			T		
pET-34b(+)		●		●	●	C			I		N						T,E		●
pET-35b(+)		●		●	●	C			I		N						T,X		●
pET-36b(+)		●		●	●	C			I		N						T,E	●	●
pET-37b(+)		●		●	●	C			I		N						T,X	●	●
pET-38b(+)		●		●	●	C			I		C						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-42a-c(+)		●		●	●	I,C			I							N	T,X		
pSCREEN-1b(+)	●		●		●	I		N	I								T,E		

Notes:
T7•Tag^{11} = 11 aa fusion tag T7•Tag^{260} = 260 aa fusion tag signal seq. = signal sequence for potential periplasmic localization
I = internal tag N = N-terminal tag C = optional C-terminal tag
protease cleavage sites: T = thrombin E = enterokinase X = Factor Xa
LIC = ligation independent cloning, vectors available separately as linearized DNA
pSCREEN-1b(+) carries the pUC origin of replication; all other pET vectors carry the pBR322 origin

G. 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 containing the T7 RNA polymerase gene, this approach is not recommended as a general strategy due to the possibility of difficulties in growth and plasmid instability that can be caused by even low levels of basal expression.

Suitable bacterial hosts for cloning include the *E. coli* K12 strains NovaBlue, JM109, and DH5α. These strains are convenient hosts for initial cloning of target DNA into pET vectors and for maintaining plasmids because they are *recA*$^-$ *endA*$^-$ and give high transformation efficiencies and good plasmid yields. NovaBlue has the additional advantage of having an 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).

H. Hosts for Expression

For protein production, a recombinant plasmid is transferred to host *E. coli* strains containing a chromosomal copy of the gene for T7 RNA polymerase. 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 (Studier and Moffatt, 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 isopropyl-β-D-thiogalactopyranoside (IPTG). Addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid.

The table on the following page lists the genotypes of strains commonly used for cloning and expression with the pET System; they are available from Novagen as competent cells ready for transformation. A complete list of catalog numbers for host strain glycerol stocks and competent cells is on p. 11.

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pET System Host Strain Characteristics

Strain	Genotype	Description/Application	Antibiotic Resistance[1]
NovaBlue	*endA1 hsdR17*($r_{K12}^- m_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proA*$^+$*B*$^+$ *lacI*^{q}*ZΔ M15* ::Tn*10*]	non-expression[2] host, general purpose cloning, plasmid preps	Tetracycline (12.5 µg/ml)
NovaBlue(DE3)	*endA1 hsdR17*($r_{K12}^- m_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proA*$^+$*B*$^+$ *lacI*^{q}*ZΔ M15* ::Tn*10*] (DE3)	*recA*$^-$ *endA*$^-$ K-12 *lacI*^{q} expression[3] host recommended for use with NovaTope System	Tetracycline (12.5 µg/ml)
BL21	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm*	control non-expression host	none
BL21(DE3)	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3)	general purpose expression host	none
BL21(DE3)pLysS	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3) pLysS	high-stringency[4] expression host	Chloramphenicol (34 µg/ml)
BL21*trxB*(DE3)	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm trxB*15::kan (DE3)	general purpose expression[3] host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml)
BL21*trxB*(DE3)pLysS	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm trxB*15::kan (DE3) pLysS	high-stringency[4] expression host: allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
BLR	F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm Δ* (*srl-recA*)*306*::Tn*10*	*recA*$^-$ non-expression host recommended for use with tandem repeats	Tetracycline (12.5 µg/ml)
BLR(DE3)	F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm Δ* (*srl-recA*)*306*::Tn*10* (DE3)	*recA*$^-$ expression host recommended for use with tandem repeats	Tetracycline (12.5 µg/ml)
BLR(DE3)pLysS	F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm Δ* (*srl-recA*)*306*::Tn*10* (DE3) pLysS	*recA*$^-$ high-stringency expression host recommended for use with tandem repeats	Chloramphenicol (34 µg/ml) Tetracycline (12.5 µg/ml)
HMS174	F$^-$ *recA hsdR*($r_{K12}^- m_{K12}^+$) *Rif*^{R}	control non-expression[2] host	Rifampicin (200 µg/ml)
HMS174(DE3)	F$^-$ *recA hsdR*($r_{K12}^- m_{K12}^+$) *Rif*^{R} (DE3)	*recA*$^-$ K-12 expression host	Rifampicin (200 µg/ml)
HMS174(DE3)pLysS	F$^-$ *recA hsdR*($r_{K12}^- m_{K12}^+$) *Rif*^{R} (DE3) pLysS	*recA*$^-$ K-12 high-stringency expression host	Chloramphenicol (34 µg/ml) Rifampicin (200 µg/ml)
AD494	*Δ ara*$^-$ *leu7697 Δ lacX74 Δ phoAPvuII phoR Δ malF3* F'[*lac*$^+$(*lacI*^{q})*pro*] *trxB*::kan	*trxB*$^-$ non-expression[2] host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml)
AD494(DE3)	*Δ ara*$^-$ *leu7697 Δ lacX74 Δ phoAPvuII phoR Δ malF3* F'[*lac*$^+$(*lacI*^{q})*pro*] *trxB*::kan(DE3)	*trxB*$^-$ expression host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml)
AD494(DE3)pLysS	*Δ ara*$^-$ *leu7967 Δ lacX74 Δ phoAPvuII phoR Δ malF3* F'[*lac*$^+$(*lacI*^{q})*pro*] *trxB*::kan(DE3) pLysS	*trxB*$^-$ high-stringency expression host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
B834	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm met*	*met* auxotroph, parent of BL21, control non-expression[2] host	none
B834(DE3)	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm met* (DE3)	met auxotroph parent of BL21, general expression host, ^{35}S-met labeling	none
B834(DE3)pLysS	F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm met* (DE3) pLysS	met auxotroph, parent of BL21, high-stringency expression host, ^{35}S-met labeling	Chloramphenicol (34 µg/ml)

1. The appropriate drug to select for the target plasmid must also be added.
2. 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 λCE6 for pET expression.
3. Expression means that the strain is a λDE3 lysogen, i.e., it carries the gene for T7 RNA polymerase under *lacUV5* control. It is therefore suited to expression from T7 promoters.
4. High-stringency means that the strain carries pLysS, a pET-compatible plasmid that produces T7 lysozyme, thereby reducing basal expression of target genes. Even greater stringency is provided by pLysE hosts; these are available separately as glycerol stocks.

I. Selecting Host Strains

A λDE3 lysogen of strain BL21 is the most widely used host for target gene expression. As an *E. coli* B strain, BL21 is deficient in the *lon* protease and lacks the *ompT* outer membrane protease that can degrade proteins during purification (Grodberg and Dunn, 1988). Thus, at least some target proteins should be more stable in BL21 than in host strains that contain these proteases. Because BL21 is sensitive to rifampicin, it is also possible to inhibit transcription by the host RNA polymerase in cases where a reduction of background synthesis of host RNA and proteins may be desirable. However, some target genes are more stable in HMS174(DE3) for reasons that are unclear. BL21 expression strains are supplied with the pET Systems and the HMS174 equivalents are available separately. In addition, Novagen offers the λDE3 Lysogenization Kit, which allows the conversion of other *E. coli* strains to DE3 lysogens.

Also available separately are strains B834, B834(DE3), and B834(DE3)pLysS, which are the BL21 parental methionine auxotrophs. BLR, BLR(DE3), and BLR(DE3)pLysS are also available, which are *recA*$^-$ derivatives of BL21 constructed by A. Roca, University of Wisconsin. B834 strains are useful for higher specific activity ^{35}S-met labeling and selenomethioninyl labeling for crystallography (Leahy et al., 1992; Wood, 1966). A recent report demonstrates significantly higher production of several target proteins in B834(DE3) than in BL21(DE3), which suggests that there may be other advantages to using the parental strain (Doherty et al., 1995). BLR strains may allow better stabilization of target genes containing repetitive sequences. In addition, the BLR strains may be more stable as λDE3 lysogens because there is no RecA protein to cleave the λ repressor.

The thioredoxin reductase-deficient strains AD494(DE3) and BL21*trxB*(DE3) are used to maximize soluble protein expression with the pET vectors. The *trxB*$^-$ cells have been shown to permit disulfide bond formation in the cytoplasm of *E. coli* (Derman et al., 1993), which also appears to depend on the presence of an oxidized form of thioredoxin (Stewart et al. 1998). Thus, the vector/host combination of pET-32 with AD494 or BL21*trxB* strains is recommended for expression of soluble proteins containing disulfide bonds. Since the *trxB* mutation in AD494(DE3) and BL21*trxB*(DE3) is maintained by kanamycin selection, these strains are not appropriate for expression of target genes cloned in kanamycin resistant pET plasmids.

Another DE3 lysogen that is used with the NovaTope® System and which may provide additional advantages for other applications is NovaBlue(DE3). This strain carries the *lacI*^{q} repressor, which provides tighter control over basal expression than the wild type repressor in the other strains. This host is especially well-suited for use with the higher copy number pSCREEN vector.

It should be noted that several popular commercial vectors carry T7 promoters and in principle could be used with the pET expression hosts. However, vectors that carry a T7 promoter without an additional source of *lac* repressor are inappropriate because multiple copies of the operator will titrate repressor and partially induce the gene for T7 RNA polymerase, which is also controlled by *lac* repressor. As a result, basal polymerase activity becomes high enough that many target genes cannot be stably maintained.

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

List of pET System Host Strains and Lambda Phages

Strain	Glycerol Stock	Competent Cells
AD494	69032-3	0.4 ml 69033-3 1.0 ml 69033-4
AD494(DE3)	69020-3	0.4 ml 69013-3 1.0 ml 69013-4
AD494(DE3)pLysS	69021-3	0.4 ml 69014-3 1.0 ml 69014-4
B834	69287-3	
B834(DE3)	69288-3	0.4 ml 69041-3 1.0 ml 69041-4
B834(DE3)pLysS	69289-3	0.4 ml 69042-3 1.0 ml 69042-4
BL21	69386-3	0.4 ml 69449-3 1.0 ml 69449-4
BL21(DE3)	69387-3	0.4 ml 69450-3 1.0 ml 69450-4 Singles 11 rxn 70235-3 Singles 22 rxn 70235-4
BL21(DE3)pLysS	69388-3	0.4 ml 69451-3 1.0 ml 69451-4 Singles 11 rxn 70236-3 Singles 22 rxn 70236-4
BL21(DE3)pLysE	69389-3	
BL21*trxB*(DE3)	70506-3	0.4 ml 70508-3 1.0 ml 70508-4
BL21*trxB*(DE3)pLysS	70507-3	0.4 ml 70509-3 1.0 ml 70509-4
BLR	69207-3	0.4 ml 69052-3 1.0 ml 69052-4
BLR(DE3)	69208-3	0.4 ml 69053-3 1.0 ml 69053-4
BLR(DE3)pLysS	69209-3	0.4 ml 69956-3 1.0 ml 69956-4
HMS174	69385-3	0.4 ml 69452-3 1.0 ml 69452-4
HMS174(DE3)	69391-3	0.4 ml 69453-3 1.0 ml 69453-4
HMS174(DE3)pLysS	69392-3	0.4 ml 69454-3 1.0 ml 69454-4
HMS174(DE3)pLysE	69393-3	
NovaBlue	69009-3	0.4 ml 69825-3 1.0 ml 69825-4 Singles 11 rxn 70181-3 Singles 22 rxn 70181-4
NovaBlue(DE3)		0.4 ml 69284-3 1.0 ml 69284-4
Bacteriophage CE6	Lysate 0.2 ml 69390-3 Lysate 10 ml 69390-4	
λDE3 Lysogenization Kit	Kit 10 rxn 69734-3	
λDE3 Lysogenization Kit plus pLysS & pLysE	Kit 10 rxn 69725-3	

J. Media Containing Glucose

In contrast to the wild type *lac* promoter, the *lacUV5* promoter that controls expression of T7 RNA polymerase in λDE3 lysogens is relatively insensitive to cAMP/CRP stimulation (Eron and Block, 1971). However, more recently it has been demonstrated that when λDE3 hosts are grown to stationary phase in media lacking glucose, cAMP mediated derepression of both the wild type and *lacUV5* promoters occurs (Grossman et al., 1998). Although growing λDE3 hosts to stationary phase is not recommended, derepression can be effectively avoided in overnight (16 h) cultures by growing λDE3 hosts in media containing 1.0% glucose, which inhibits cAMP production. Typically, glucose addition is not required to maintain plasmid stability; however, in cases of extremely toxic target genes the addition of glucose to both agar plates and liquid media should help decrease basal expression. Note that addition of glucose is neither necessary nor recommended during the cloning steps in non-expression hosts.

K. The T7*lac* Promoter

Even in the absence of IPTG, there is some expression of T7 RNA polymerase from the *lacUV5* promoter in λDE3 lysogens. If target gene products are sufficiently toxic to *E. coli*, this basal level can be enough to prevent the establishment of plasmids in λDE3 lysogens. Several solutions to this problem are available with the pET System.

One approach is to use vectors that contain what is termed a T7*lac* promoter (Studier et al., 1990; Dubendorff and Studier, 1991; see table on p. 7). 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 T7*lac* and *lacI* promoters diverge. When this type of vector is used in DE3 lysogens to express target genes, 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 T7*lac* 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 BL21(DE3) or HMS174(DE3) (Dubendorff and Studier, 1991).

L. pLysS and pLysE Hosts

One way of providing additional stability to target genes is to express them in host strains containing a compatible plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase (Moffatt and Studier, 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. When produced from the cloned gene, relatively high levels of T7 lysozyme can be tolerated by *E. coli*, apparently because the protein is unable to pass though the inner membrane to reach the peptidoglycan layer. Treatments that disrupt the inner membrane but do not normally cause lysis, such as freeze-thaw, the addition of chloroform, or mild detergents (e.g., 0.1% Triton X-100), induce rapid lysis of cells that contain even small amounts of T7 lysozyme.

T7 lysozyme is provided to the cell from a clone of the T7 lysozyme gene in the *Bam*H I site of pACYC184 (Chang and Cohen, 1978). The cloned fragment (bp 10,665-11,296 of T7 DNA; Dunn and Studier, 1983) also contains the ϕ3.8 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. These plasmids confer resistance to chloramphenicol and are compatible with the pET vectors for cloning target genes. 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.

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 ϕ3.8 promoter to make lysozyme mRNA. However, the ϕ3.8 promoter is relatively weak [McAllister et al. 1981], and most transcription should be from the much stronger ϕ10 promoter used in the target plasmids.) When using the T7*lac* promoter, we have observed that expression in pLysS hosts can be somewhat reduced relative to non-pLysS hosts under a given induction condition.

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. (In contrast, the high level of expression in the absence of lysozyme or in the presence of pLysS usually prevents continued growth of the cell.)

The presence of either pLysS or pLysE increases the tolerance of λDE3 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 T7*lac* promoter-containing vector and pLysS is preferable.

The presence of pLysS (or pLysE) has the further advantage of facilitating the preparation of 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 X-100, will allow the resident T7 lysozyme to efficiently lyse the cells. This property can make it advantageous to carry pLysS in the cell even when it is not required for stabilizing the target plasmid.

M. Bacteriophage CE6

A final alternative for toxic genes is to introduce the 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 and Moffatt, 1986). When CE6 infects HMS174, 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. Bacteriophage CE6 is available separately from Novagen (see p. 11).

N. 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 testing of performance. The strain is provided as a glycerol stock of an appropriate λDE3 lysogen containing a pET plasmid with an insert encoding β-galactosidase, which can be easily assayed spectrophotometrically (except for Controls H, J, L, and N, which contain no insert). The following table lists the various induction control strains and matching pET vectors. See *Induction Control* on p. 45 for more details on the β-galactosidase assay.

Control	Vector	Host strain	Selection	Promoter	N-terminal fusion	Protease site	Insert (protein size)	Included with vector/series	Cat. No.
A	pET-14b	BL21(DE3)pLysS	amp cam	T7	His•Tag	T	β-gal 118kDa	pET-3, 5, 12, 14b, 17b, 17xb, 20b, 23	69674-3
B	pET-15b	BL21(DE3)pLysS	amp cam	T7*lac*	His•Tag	T	β-gal 118kDa	pET-11, 15b, 21, 22b, 25b	69257-3
C	pET-16b	BL21(DE3)pLysS	amp cam	T7*lac*	His•Tag	X	β-gal 119kDa	pET-16b	69675-3
D	pET-19b	BL21(DE3)pLysS	amp cam	T7*lac*	His•Tag	E	β-gal 119kDa	pET-19b	69676-3
E	pET-28b(+)	BL21(DE3)	kan	T7*lac*	His•Tag T7•Tag	T	β-gal 119kDa	pET-9, 24, 26b, 27b, 28	69258-3
F	pET-29b(+)	BL21(DE3)	kan	T7*lac*	S•Tag	T	β-gal 119kDa	pET-29	69259-3
G	pET-30b(+)	BL21(DE3)	kan	T7*lac*	His•Tag S•Tag	T, E	β-gal 121kDa	pET-30	69554-3
H	pET-31b(+)	BLR(DE3)pLysS	amp cam, tet	T7*lac*	KSI		none 14.8kDa	pET-31b	69966-3
J	pET-32a(+)	BL21(DE3)	amp	T7*lac*	Trx•Tag His•Tag S•Tag	T, E	none 20.4kDa	pET-32	69030-3
K	pET-34b(+)	BL21(DE3)	kan	T7*lac*	CBD_{clos}•Tag S•Tag	T, E	β-gal 138kDa	pET-34b, 35b, 36b, 37b, 38b	70125-3
L	pET-39b(+)	BL21(DE3)	kan	T7*lac*	DsbA His•Tag S•Tag	T, E	none 32.2kDa	pET-39b, 40b	70463-3
M	pET-33b(+)	BL21(DE3)	kan	T7*lac*	His•Tag PKA site T7•Tag	T	β-gal 120kDa	pET-33b	70514-3
N	pET-41b(+)	BL21(DE3)	kan	T7*lac*	GST•Tag His•Tag S•Tag	T, E	none 35.6kDa	pET-41, 42	70535-3

Abbreviations: amp = ampicillin or carbenicillin, kan = kanamycin, cam = chloramphenicol, tet = tetracycline
T = thrombin, X = Factor Xa, E = enterokinase

II. Getting Started

A. The pET System Process

Process	Detail	Products
Prepare pET Vector	1. Digest with restriction enzyme(s) and desphosphorylate, or use LIC vector 2. Gel purify (or use LIC vector)	• pET Vector DNA • pET LIC Vector Kits • PCR Markers • Perfect DNA™ Markers
Prepare Insert DNA	1. Plasmid prep and/or PCR 2. Restriction digest or generate LIC overhangs 3. Gel purify	• pET LIC Vector Kits • PCR Markers • Perfect DNA Markers
Clone Insert into pET Vector	1. Ligate or anneal insert with pET vector 2. Transform into non-expression host (e.g. NovaBlue) 3. Identify positive clones; colony PCR, miniprep, verify reading frame by sequencing, or *in vitro* transcription/translation	• Clonables™ Kit • DNA Ligation Kit • NovaBlue Competent Cells • NovaBlue Singles™ Competent Cells • Single Tube Protein™ System 3, T7 • Vector primers • Antibiotics (see p. 17)
Transform into Expression Host	Transform host carrying T7 RNA polymerase gene (λDE3 lysogen) or non-DE3 host compatible with λCE6 infection	• Expression host competent cells • BL21(DE3) Singles Competent Cells • BL21(DE3)pLysS Singles Competent Cells • λCE6
Induce and Optimize Expression of Target Protein	1. Test plasmid stability 2. Determine time course and temperature for expression in total cell and subcellular fractions; analyze solubility and activity 3. Detect target protein by SDS-PAGE, Western blot, quantitative assay	• BugBuster™ Protein Extraction Reagent and Purification Kits • CBINDT™ Resins and Cartridges • CBINDT Buffer Kit • CBD•Tag™ Antibodies • GST Assay Kit • GST•Tag™ Antibody • GST•Bind™ Resin and Buffer Kit • His•Bind® Resin and Buffer Kit • His•Bind Quick Cartridges and Columns • HSV•Tag® Antibody • Protease Inhibitor Cocktails • Protein Refolding Kit • S•Tag™ Rapid Assay Kit • S•Tag Western Blot Kits • S•Tag Purification Kits • T7•Tag® Antibody and Conjugates • T7•Tag Affinity Purification Kit • Restriction Grade Thrombin, Biotinylated Thrombin, Factor Xa, rEnterokinase
Scale-up	1. Scale up culture 2. Prepare extract 3. Affinity purify 4. Cleave tags and remove protease (if desired)	
Purify Target Protein		

B. Growth Media

A wide range of growth media is suitable for growth of strains and expression of target DNAs in the pET System. Suitable growth media are M9, M9ZB, LB broth, and TB ("terrific broth"). Recipes and stock solutions are shown below and on the following page.

LB*	**M9**	**M9ZB** (Studier et al., 1990)
Per liter: 10 g Bacto tryptone 5 g Yeast extract 10 g NaCl • Adjust pH to 7.5 with 1N NaOH • Autoclave	Per liter: 50 ml 20X M9 salts 20 ml 20% glucose 1 ml 1 M $MgSO_4$ 0.5 g NaCl 930 ml autoclaved deionized H_2O	Per liter: 10 g N-Z-amine A (Quest) 5 g NaCl • Autoclave and cool • Add 100 ml 10X M9 salts, 1 ml 1M $MgSO_4$, 10 ml 40% glucose (from autoclaved stocks)

10X M9 salts	**20X M9 salts**	**K phosphate**
Per liter: 10 g NH_4Cl 30 g KH_2PO_4 60 g $Na_2HPO_4 \bullet 7H_2O$ • Autoclave	Per liter: 20 g NH_4Cl 60 g KH_2PO_4 120 g $Na_2HPO_4 \bullet 7H_2O$ • Autoclave	Per liter: 23.1 g KH_2PO_4 125.4 g K_2HPO_4 • Autoclave

TB* (Sambrook et al., 1989)
Per liter: 900 ml deionized water 12 g Bacto tryptone 24 g Yeast extract 4 ml glycerol • Autoclave, cool to 60°C • Add 100 ml sterile K phosphate

* Add glucose to 1% final concentration using an autoclaved 20% stock when dealing with toxic genes (see p. 12).

Stock solution	Preparation
100 mM IPTG (isopropyl β-D-thiogalactopyranoside) *Cat. No. 70527-3*	2.38 g IPTG in 100 ml deionized water. Filter sterilize, and store at –20°C.
Carbenicillin (disodium salt) *Cat. No. 69101-3*	50 mg/ml in deionized water. Store at –20°C. Use at 50 µg/ml.
Ampicillin (sodium salt)	25 mg/ml in deionized water. Store at –20°C. Use at 50 µg/ml.
Chloramphenicol *Cat. No. 220551*	34 mg/ml in ethanol. Store at –20°C. Use at 34 µg/ml.
Kanamycin (sulfate) *Cat. No. 420311*	30 mg/ml in deionized water. Store at –20°C. Use at 30 µg/ml for cells containing kan^R plasmids, and at 15 µg/ml for cells with a chromosomal kan^R gene (AD494, BL21*trxB*).
Tetracycline *Cat. No. 58346*	12.5 mg/ml in ethanol. Store at –20°C. Use at 12.5 µg/ml.
Rifampicin *Calbiochem # 557303*	10 mg/ml in 67% methanol, 0.17 N NaOH. Use at 200 µg/ml within 5 days. Protect from light.

C. Storage of Strains

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

To prepare stock cultures of host strains and pET recombinants:

1. Inoculate a single colony into 50 ml medium containing appropriate antibiotic(s) in a 250 ml flask.
2. Incubate with vigorous shaking at 37°C during the day until the OD_{600} reaches 0.6–0.8.
3. Remove 0.9 ml and transfer to a cryovial, add 0.1 volume of 80% glycerol.
4. Mix well and store at –70°C.

Plasmid-bearing strains, particularly those having any tendency toward instability, are titered at the time of freezing to be sure that the vast majority of cells in the culture have the intended host-plasmid combination (see *Optimizing Expression*, p. 27).

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 antibiotic).
3. Return the remainder to the –70°C freezer without thawing.

D. Vector Preparation

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.

Recommendations

- Note that different enzymes cut 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.

Note: *As described in Section I, About the System, it is also possible to clone PCR products and other fragments without restriction digestion using the ligation-independent cloning (LIC) method with Novagen's Ek/LIC and Xa/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 digestion to decrease the background of non-recombinants due to 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 prior to insert ligation to remove residual nicked and supercoiled plasmid, which transform very efficiently relative to the desired ligation products. This step is optional but it usually reduces the effort required to screen for the correct construction.

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	Units for each restriction enzyme (assuming compatible buffer; 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 brought to volume
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 check the extent of digestion.
4. When digestion is complete, add calf intestinal alkaline phosphatase directly to the remainder of the digestion. The 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/or can be difficult to remove for future steps. Three 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 units of alkaline phosphatase per pmol ends. If necessary, 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 cut the band from the gel using a clean razor blade.
8. Recover the DNA from the gel slice using your favorite method. The method chosen should also remove residual alkaline phosphatase activity without the need for phenol extractions. Resuspend the final product in a total volume of 30 µl (usually about 50 ng/µl DNA). Assume recoveries in the range of 50% for the ligation step.
9. Store the treated vector at –20°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 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 x g for 10 min, rinse the pellet with 70% ethanol, air dry, and resuspend in 30 µl TE buffer.*

E. Insert Preparation

Preparing inserts by restriction digestion followed by gel purification is 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, (3) add LIC overhangs, and (4) 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 about 50%, and restriction sites should be flanked by 3–10 (depending on the enzyme) "spacer" nucleotides 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 reaction can be minimized in several ways:

- Use an enzyme with proofreading activity, such as *ULTma* DNA polymerase (Perkin-Elmer).
- Limit the number of PCR cycles.
- Increase the concentration of target DNA.
- Increase the primer concentration.

Important: *To achieve efficient amplification of entire CBD• Tag sequences in pET CBD vectors, glycerol must be added to bring the PCR reaction mixture to a final concentration of 8–10%. This is not required when using primers that amplify only a small portion of a CBD, i.e., CBD_{clos}• Tag, CBD_{cenA}• Tag, and ASCBDcex primers. Note that the addition of glycerol is also required when performing Single Tube Protein System 3 (STP3) analysis of CBD• Tag constructs with the pET upstream or T7 promoter primers.*

III. Cloning Inserts in pET Vectors

Procedures and recommendations in this section cover the process of cloning your insert into the pET vector. This process includes ligation and transformation into a non-expression host, and analyzing your construct. Novagen's Clonables™ Kit (Cat. No. 70256-3) contains pretested ligation mix and highly efficient competent cells designed for convenient, reproducible ligation and transformation of vector and insert having any type of end. Please refer to TB233 (supplied with the Clonables Kit) for the protocol. After the construct is verified, plasmid is transformed into an expression host for protein production.

A. Ligation

One consistently successful protocol for ligation is presented here.

Sample ligation protocol

1. 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 (50 ng of a 500 bp fragment) in a volume of 20 µl. Assemble the following components in a 1.5 ml tube (these components are available separately in the DNA Ligation Kit, Cat. No. 69838-3). Add the ligase last.

2 µl	10X Ligase Buffer (200 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$, 250 µg/ml acetylated BSA)
2 µl	100 mM DTT
1 µl	10 mM ATP
2 µl	50 ng/µl prepared pET vector
1 µl	T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
x µl	Prepared target gene insert (0.2 pmol)
y µl	Nuclease-free water to volume
20 µl	Total volume

2. Add the ligase last, and 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

Initial cloning should be done in a *recA*$^-$ cloning strain, such as NovaBlue, or other similar host that lacks the gene for T7 RNA polymerase. This enables high percentage monomer plasmid yields for examination of the construct sequence, as well as separation of cloning from expression. This separation can be valuable in troubleshooting any difficulties that might arise during later procedures.

The strains described above for cloning and expression with pET vectors can be prepared for transformation by standard procedures. Expect BL21 and its derivatives to be transformed at about 1/10 the efficiency of the other strains. For convenience and consistent performance, Novagen offers the relevant host strains as prepared competent cells, ready for high-efficiency transformation (see pp. 9 and 11).

DNA in ligation reactions containing high-quality reagents is suitable for direct addition to Novagen's Competent Cells (no more than 1 µl ligation should be used per 20 µl cells). Inactivation of the ligase is not required prior to transformation. 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 pH 8.0, 1 mM EDTA) or in water.

Novagen's Competent Cells are provided in 0.2 ml aliquots, except for NovaBlue, BL21(DE3) and BL21(DE3)pLysS Singles Competent Cells, which are provided in 50 µl aliquots (see TB009 for the recommended transformation protocol for Singles). The following protocol should be used with the 0.2 ml aliquots.

United States & Canada
Orders: 800 526-7319
Technical Service: 800 207-0144

Handling Tips

1. Upon receipt from Novagen, 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°C or below. For optimal results, do not allow the cells to thaw at any time prior to use.
2. Handle only the very top of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible.
3. To mix cells, flick the tube 1–3 times. *NEVER* vortex the competent cells.
4. To avoid multiple freeze-thaw cycles of the standard 0.2 ml cells, dispense the cells into aliquots after the initial thaw and store them at –70°C or below (note that Singles cells are provided as 50 µl aliquots, which are used "as is" and do not require dispensing). To dispense aliquots of cells from the 0.2 ml stock, remove the stock tube quickly from the ice and flick 1–2 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. Place the aliquot immediately into the bottom of a pre-chilled 1.5 ml tube, mix by pipetting once up and down, and then immediately close the tube and replace on ice. After all of the aliquots are taken, return any unused tubes to the freezer before proceeding with the transformation.

Procedure

Tip: *Prepare LB agar plates with appropriate antibiotic ahead of time (see step 10).*

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 surrounded by ice. Also, place the required number of empty 1.5 ml polypropylene microcentrifuge tubes on ice to pre-chill. Allow the cells to thaw on ice for ~2–5 min.
2. Visually check the cells to see that they have thawed and gently flick the cells 1–2 times to evenly resuspend the cells. Pipet 20 µl aliquots of cells into each of the pre-chilled tubes.
3. (Optional) To determine transformation efficiency, add 0.2 ng supercoiled plasmid (or Test Plasmid provided with Competent Cells) in a volume of 1 µl to one of the tubes containing cells. Stir gently to mix.
4. 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, making sure that the tube is surrounded by ice except for the cap. Repeat for additional samples.
5. Place the tubes on ice for 5 min.
6. Heat the tubes for exactly 30 sec in a 42°C water bath; do not shake.
7. Place the tubes on ice for 2 min.
8. Add 80 µl of room temperature SOC medium to each tube.
9. **When using NovaBlue**: if selecting for β-lactamase ($carb^R$/amp^R), no outgrowth (shaking incubation) step is required, although slightly higher cloning efficiencies may be obtained with 30–60 min outgrowth. Plate 5-50 µl cells directly on selective media (Step 10). If selecting for kanamycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating on selective media.

   When using strains other than NovaBlue: shake at 37°C (250 rpm) for 60 min prior to plating on selective media. If selecting for ampicillin resistance, cells can be plated directly with no outgrowth period, but colony yield will be 10–50-fold lower. Outgrowth is always recommended when using kanamycin selection. Shake at 200–250 rpm at 37°C for 1 h.

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 x 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 ~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 ~20 min prior to plating.

10. Spread 50 µl of each transformation (5 µl for supercoiled test plasmids) on LB agar plates containing the appropriate antibiotic. Use 50 µg/ml carbenicillin or ampicillin for amp^R

vectors, or 30 µg/ml kanamycin for vectors having the kan^R gene. The plates should also contain 34 µg/ml chloramphenicol if strains carrying pLysS or pLysE are used. When plating less than 25 µl, first pipet a "pool" of SOC onto the plate and then pipet the cells into the SOC. Please see the next section for additional details on plating technique.

Important: *The appropriate amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. As little as 2 µl will yield several hundred transformants under highly efficient conditions (e.g., with NovaBlue cells giving > 4 x 10^8 cfu/µg). For recombinants in NovaBlue, expect 10^5–10^7 transformants/µg plasmid, depending on the particular insert and the ligation efficiency.*

***When using the Test Plasmid**, plate no more than 5 µl of the final transformation mix (e.g., 5 µl of NovaBlue cells at 1 × 10^8 efficiency) in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the amp^R gene).*

11. Let the plates sit on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

Plating Technique

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, flick the transformation tube 5–8 times, open the cap and immediately remove the sample volume from the middle of the transformation reaction.
3. Transfer the sample to the plate by dispensing the sample volume into the SOC cushion. After the sample is out of the pipet tip, use the same tip to pipet up the sample volume's worth of SOC from the cushion edge and dispense that SOC back into the cushion. (This effectively rinses out your pipet tip.)
4. 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 ~10 sec prior to placing the spreader on the plate. Place the spreader on the LB agar at the outside of the plate (not touching the pool of cells). This further cools the spreader on the LB agar before spreading the cells.
5. Slowly turn the plate while supporting the weight of the spreader.

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

6. 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. Once the moisture is absorbed, do not continue spreading. If the plates are wet, spread until the sample is evenly distributed. Do not spread until the sample and cushion have absorbed completely into the plate, as overspreading can decrease transformation efficiency. Instead, after spreading briefly, allow the plates to sit upright at room temperature for ~15 min prior to placing them in the 37ºC incubator. This will allow excess moisture to absorb into the plates before the plates are inverted and placed in the incubator (step 7).
7. Incubate all plates, cover-side down, in the 37ºC 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). Once the colonies are at the desired size, the plates can be placed at 4ºC.

C. 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 can be successful even though the number of colonies on the two plates are roughly equivalent. There are several methods for analysis of transformants, including colony PCR, plasmid miniprep and restriction analysis, sequencing, and *in vitro* transcription and translation.

Prior to growing colonies for plasmid isolation, the presence of the appropriate insert as well as 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 µl) of one of the vector-specific primers is used

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with 5 pmol of one of the original insert-specific PCR primers in two separate reactions 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 almost all pET vectors. Alternatively, just the vector-specific primers can be used in one reaction if insert orientation information is not desired.

Transcription/Translation Analysis of PCR Products with STP3™

pET constructs can be quickly evaluated for expression of the desired target protein using the Single Tube Protein System 3, T7 (STP3, T7; Cat. No. 70192-3) in which PCR-amplified DNA is directly transcribed and translated *in vitro*. The PCR step can be performed with ligations of vector plus insert, or with colonies from the transformation. The colony procedure is especially useful when target sequences are cloned via PCR, because it allows rapid screening for PCR-related introduction of unwanted stop codons in individual clones.

To prepare suitable templates for transcription/translation, use the appropriate primers, which allow amplification of the T7 promoter along with the correct orientation of the target insert. The T7 promoter primer is *not* suitable for this application because T7 RNA polymerase does not transcribe efficiently if the promoter is at the very end of the molecule. The pET upstream primer (Cat. No. 69214-3) is an appropriate 5' primer for all pET vectors except pET-17b, 17xb, 20b(+), 23(+), 23a-d(+), pEX*lox*® and pSCREEN. By using an insert-specific 3' primer, the correct orientation of insert is preferentially amplified, which is necessary if the ligation is into a single restriction site (i.e., non-directional). The T7 terminator primer is appropriate as the 3' primer for non-orientation specific amplification of target genes in all pET vectors except the pET-5 series.

Under most conditions, 1–2 µl of the PCR reaction can be used directly in the transcription/translation reaction without further purification (see the Single Tube Protein System 3 protocol, TB206, for further details).

Ligation PCR for STP3 Analysis

1. Assemble the following components for ligation PCR:

1 µl	Ligation reaction diluted 1:10 in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) (0.25–0.5 ng vector)
5 µl	10X Buffer (10X = 100 mM Tris-HCl pH 8.8 at 25°C, 500 mM KCl, 1% Triton X-100)
1 µl	pET upstream primer (5 pmol)
1 µl	downstream primer (5 pmol)
3 µl	25 mM $MgCl_2$
1 µl	10 mM dNTP Mix
0.25 µl	(1.25 U) AmpliTaq® DNA polymerase (Perkin-Elmer)
x µl	Sterile deionized water to volume
50 µl	Total volume

2. Add the enzyme or DNA last to start the reaction, mix gently, and add 2 drops of mineral oil from a 200 µl 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.
3. Insert the tubes in a Perkin-Elmer thermal cycler and carry out the PCR for 30 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
4. To remove the oil overlay and inactivate the polymerase, add 100 µl of chloroform, mix 30 seconds, and centrifuge for 1 min. The top aqueous phase (which may appear cloudy) contains the DNA products. If desired, remove a 5–10 µl sample for gel analysis (refer to *Colony Screening* for details). A strong band should appear that corresponds to the distance between (and including) the primers.
5. Add 2 µl of the aqueous phase directly to a Single Tube Protein System 3 reaction for protein synthesis.

Colony PCR for STP3 Analysis

1. Pick a colony from an agar plate using a 200 µl 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 the bulk of the colony to the tube in the next step.
2. Transfer the bacteria to a 0.5 ml tube containing 50 µl 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 × g for 1 min to remove cell debris.
5. Transfer 10 µl of the supernatant to a fresh 0.5 ml tube for PCR. Leave on ice until use.
6. Make a master reaction mix by combining the following. (Note that amounts given are per reaction.)

   Per reaction:

28.8 µl	sterile water
1 µl	dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
1 µl	pET upstream primer, 5 pmol/µl
1 µl	downstream primer, 5 pmol/µl
5 µl	10X Buffer (10X = 100 mM Tris-HCl pH 8.8 at 25°C, 500 mM KCl, 1% Triton X-100)
3 µl	25 mM $MgCl_2$
0.25 µl	(1.25 U) AmpliTaq DNA polymerase (Perkin-Elmer)

   Mix the above components together in a single tube using amounts corresponding to the number of reactions desired. To account for pipetting losses, it is convenient to multiply the amounts by X.5, where X is the number of reactions.
7. Add 40 µl of the master mix to each sample, mix gently, add 2 drops of mineral oil, cap the tubes and put the samples in a thermal cycler (Perkin-Elmer).

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 µl of chloroform, mix 30 seconds, and centrifuge for 1 min. The top aqueous phase (which may appear cloudy) contains the DNA products. If desired, remove a 5–10 µl sample for gel analysis (refer to *Colony Screening* for details).
10. Add 2 µl of the reaction directly to a Single Tube Protein System 3 reaction for protein synthesis.

Colony Screening

Colonies can be screened for inserts without the need for minipreps by direct colony PCR using Novagen's vector-specific primers as described in the prior section.

For most pET vectors, appropriate primers for colony PCR without *in vitro* transcription/ translation are the T7 promoter primer and the T7 terminator primer (Cat. Nos. 69348-3 and 69337-3, respectively). Exceptions are pET-17xb, pSCREEN-1b(+) and pEX*lox*(+) vectors, in which the T7 gene *10* primer (Cat. No. 69341-3) is recommended over the T7 promoter primer; and pET-5a-c, which require the use of the pBR322 *Eco*R I clockwise primer (Cat. No. 69336-3) instead of the T7 terminator primer.

To analyze the reaction products (step 9, Colony PCR):

1. Remove the oil overlay by adding 100 µl of chloroform.
2. Add 5 µl of 10X loading dye to the top aqueous phase.

3. Load 10-25 µl per lane on a 1% agarose gel containing 0.5 µg/ml ethidium bromide together with Perfect DNA Markers.

Note: PCR products can be sequenced directly using Novagen's Strandase™ ssDNA Preparation Kit (Cat. No. 69202-3) followed by conventional single stranded sequencing methods (provided that the appropriate combination of phosphorylated primers are used in the PCR step).

Plasmid Miniprep Procedure

After positive clones are identified, the plasmids can be isolated for transformation into expression hosts, restriction mapping, and sequence analysis. The procedure yields 1–2 µg pET plasmid from 1.5 ml of culture (5–10 µg for pSCREEN and pEX*lox*, which contain the high-copy number pUC origin of replication). This is sufficient for transfer into an expression host, but not enough for other types of analysis. The protocol can be scaled up proportionally if more plasmid is needed (e.g., a 50 ml preparation will yield 35–75 µg pET plasmid).

The following protocol is a slight modification of one presented in Sambrook et al. (1989).

1. Using a sterile loop, toothpick or pipet tip, transfer a well-isolated colony into 3 ml of LB broth supplemented with the appropriate antibiotic(s), in a Falcon 2059 culture tube. Cap loosely and incubate with shaking at 37°C for 6 h to overnight.
2. Transfer 1.5 ml of culture into a 1.5 ml microcentrifuge tube and centrifuge at 12,000 × g for 1 min.
3. Remove the medium by aspiration, leaving the pellet as dry as possible.
4. Resuspend the cells in 100 µl of ice-cold 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA. Pipet up and down to make sure that the pellet is completely suspended.
5. Add 200 µl of freshly prepared 0.2 N NaOH, 1% SDS. Mix by inversion and leave on ice for 3 min.
6. Add 150 µl of ice-cold 3 M NaOAc, pH 5.2. Mix by inversion and leave on ice for 5 min.
7. Centrifuge at 12,000 × g for 5 min. Transfer the clear supernatant to a fresh tube, avoiding the pellet, which tends to break up easily. Spin again if too much particulate matter remains in the supernatant.
8. Add 400 µl TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1) and vortex for 30 sec.
9. Centrifuge at 12,000 × g for 1 min at room temperature.
10. Transfer the top aqueous phase to a fresh tube and add 800 µl ethanol. Vortex, leave at room temperature for 2 min, and centrifuge at 12,000 × g for 5 min at 4°C.
11. Decant the supernatant and add 400 µl ethanol to the pellet.
12. Spin briefly, pour off the ethanol and allow the pellet to air dry in an inverted position for about 10 min.
13. Resuspend the pellet in 30 µl of TE buffer containing 20 µg/ml RNase A and incubate at 37°C for 15 min.
14. At this point, the DNA can be used for transformation of expression hosts. However, it is advisable to check your construct prior to transforming the expression host, either by the STP3 method described on pp. 23–24 or by sequencing.

Note: RNase must be removed if you are planning to do in vitro transcription and translation. A satisfactory procedure is to add TE to 100 µl, and then extract successively with 1 volume TE-buffered phenol, 1 volume phenol:CIAA (1:1; CIAA is chloroform:isoamyl alcohol, 24:1), and 1 volume 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 place at –20°C for 30 min, centrifuge 5 min at 12,000 x g, remove the supernatant, and rinse the pellet with 70% ethanol. Dry and resuspend the DNA in 30 µl TE. If desired, 2 µl Pellet Paint™ 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).

To help make the pellet visible after precipitation, and 2 µl of Pellet Paint Co-precipitant (Cat. No. 69049-3) in step 1. Pellet Paint is compatible with cyanine dye based fluorescent sequencing methods but is not recommended for rhodamine based labeling methods (e.g. ABI automated sequencers).

Sequencing

For double stranded plasmid sequence analysis, the preparation must be further processed to remove RNA breakdown products. This can be easily accomplished by precipitation with polyethylene glycol (PEG).

PEG precipitation for sequencing:

1. Add 10 µl of 30% PEG-8000, 1.5 M NaCl (prepare from autoclaved stocks of 50% PEG and 5 M NaCl to avoid possible DNase contamination) and vortex thoroughly.
2. Incubate on ice for 20 min.
3. Centrifuge at 12,000 × g at 4°C for 10 min.
4. Carefully remove the supernatant, leaving the small transparent DNA pellet behind.
5. Rinse the pellet successively with 70% ethanol and then 100% ethanol, and let air dry.
6. Resuspend the DNA in 20 µl TE buffer. The plasmid is now suitable for alkali denaturation and double stranded sequencing.

Note: *It is possible to prepare single stranded DNA template either from PCR products with the Strandase Kit (Cat. No. 69202-3) or from pET plasmids that carry the phage f1 origin of replication (i.e., pET plasmids having a (+) designation in their name) by superinfection 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 M13KO7) 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.)*

Detailed protocols for sequencing with double stranded and single stranded templates are available from many manufacturers of sequencing kits. The following references are also useful: Chen and Seeburg, 1985; Hattori and Sakaki, 1986; Mierendorf and Pfeffer, 1987; Ausubel et al., 1989.

IV. Expressing the Target Gene

A. Expression Host Transformation

For transformation into an expression host (i.e., a λDE3 lysogen), obtain or prepare appropriate competent cells and use 1 µl of a 50-fold dilution (approx. 1 ng) of plasmid in sterile water or TE buffer and follow the transformation procedure on p. 20. Streak transformants for single colonies and prepare glycerol stocks as described on p. 17.

B. Induction of λDE3 Lysogens

After a target plasmid is established in a λDE3 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, while 1 mM IPTG is recommended with vectors having the T7*lac* promoter. An example of an induction protocol is presented below. Detailed protocols for small scale induction, fractionation and analysis of expression are given in Section VI, *Purifying Target Proteins*.

Preparation for Induction

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

Note: *Include 34 µg chloramphenicol/ml if the cells carry pLysS or pLysE.*

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

Do not let cultures grow at 37°C overnight. This can lead to derepression of the lacUV5 promoter controlling T7 RNA polymerase expression (see p. 12). In addition, prolonged incubation at stationary phase may deplete the media of antibiotic and allow overgrowth of non-recombinants or non-expressing mutants.

Sample Induction Protocol

1. Incubate with shaking at 37°C until OD_{600} reaches 0.4–1 (0.6 recommended; about 3 h).
2. Remove samples for the uninduced control and for titering as described in *Plasmid Stability Test* below. To the remainder, add IPTG from a 100 mM stock to a final concentration of 0.4 mM (T7 promoter) or 1 mM (T7*lac* promoter) and continue the incubation for 2–3 h.
3. Place the flasks on ice for 5 min and then harvest the cells by centrifugation at 5000 × g for 5 min at 4°C. Save the supernatant, if desired, for further analysis (see Section VI, *Purifying Target Proteins*, for analysis of media fraction).
4. Resuspend the cells in 0.25 culture volume of cold 20 mM Tris-HCl pH 8.0, and centrifuge as above.
5. Remove the supernatant and store the cells as a frozen pellet at –70°C or continue with purification (note that inclusion bodies become less soluble upon aging in the freezer).

Note: *The cells will lyse when thawed if they carry pLysS or pLysE.*

C. Optimizing Expression

This section describes procedures and recommendations regarding optimizing expression of your target protein. Included here are considerations for plasmid stability, protein solubility and a discussion of factors that influence target gene expression.

Plasmid Stability Test

Immediately before induction, we recommend testing the culture to determine the fraction of cells that carry the target plasmid. This involves plating on four plates that differ in the additions to the agar used in plating. Plate cells at a dilution of 10^{-5} on plates that have both IPTG and antibiotic, and just IPTG (plates 1 and 2); and plate cells at a dilution of 2×10^{-6} on plates that have just antibiotic, and nothing added to the agar (plates 3 and 4). This test and its interpretation is described more fully later in this section, in *Toxic Genes and Plasmid Instability*. (It is not usually necessary to test for the relatively stable pLysS.)

If appropriate attention is paid to the possibility of plasmid instability, more than 98% of the cells in the culture will usually contain expressible target plasmid. Cells are usually harvested 2–3 h after induction, enough time for substantial accumulation of target protein, but before the culture can be overgrown with cells that have lost plasmid or are otherwise unproductive. However, some target proteins continue to accumulate for much longer times.

D. Solubility

The optimal scheme and time course for induction can vary, because the characteristics of each target gene product are unique. For example, growth at 37°C causes some proteins to accumulate as inclusion bodies, while incubation at 30°C leads to soluble, active protein (Schein and Noteborn, 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.

In addition, 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. Factors to consider when using this approach are the critical micelle concentration of a given detergent and the detergent:protein ratio required for solubilization and delipidation of a target protein. For a review of the use of detergents in bacterial lysis, see “Experiment 2: Solubilization and Purification of the Rat Liver Insulin Receptor” (Brennan and Lin, 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 cell debris; in theory, they may be dissociated by adding salt to the lysis buffer.

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. There has been substantial progress in the last several years in methodologies for the refolding of proteins derived from inclusion bodies (for review articles, see Rudolph and Lilie, 1996; Mukhopadhyay, 1997). Many refolding schemes have been reported; optimal protocols vary with the target protein and must be determined empirically. The Protein Refolding Kit provides a convenient set of reagents and protocol to facilitate refolding of many proteins.

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 in aggregates. It should be noted that solubility does not necessarily indicate that a protein is folded properly; some proteins form soluble species that are inactive. If a significant fraction is soluble and active, the induction can be scaled up in volume, and an affinity chromatography step can be used to purify and concentrate the soluble fraction.

Formation of Disulfide Bonds: pET-32, pET-39 and pET-40

Solubility can also be manipulated by the choice of vector, cloning sites, or host cells. For example, the use of a pET-32 series vector to produce a thioredoxin fusion protein may increase the yield of soluble product in the cytoplasm (LaVallie et al., 1993, Novy et al., 1995). The use of AD494 or BL21*trxB* strains allows the formation of disulfide bonds in the *E. coli* cytoplasm, which may affect the solubility and/or activity of a given target protein. If your target protein contains one or more essential disulfide bonds, the combination of a pET-32 vector and a *trxB*$^{-}$ host may prove to be optimal because disulfide bond formation in the cytoplasm appears to be dependent on the presence of thioredoxins (Stewart et al., 1998).

In contrast to the cytoplasm, the periplasm of *E. coli* is an oxidizing environment that contains enzymes that catalyze the formation and isomerization of disulfide bonds (Rietsch et al., 1996; Raina and Missiakis, 1997; Sone et al., 1997). Directing heterologous proteins to the periplasm is a common strategy employed when attempting to isolate active, folded proteins containing disulfide bonds. Typically, a target gene is fused to a signal sequence enabling potential localization of the expressed protein to the periplasm. While a number of 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), respectively, of disulfide bonds in the periplasm (Missiakis 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). However, note that some target proteins will not be good candidates for this strategy; for example, some fusions of β-gal to a periplasmic protein have proven to be toxic (Snyder and Silhavy, 1995). Also note that over-expressed, 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 and Swartz, 1997). Typically, a DsbC fusion protein expressed from pET-40b(+) is first purified by 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^{2+}.

E. 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 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.

Use of Ampicillin

Use of ampicillin as a selective antibiotic requires special care, because β-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 the 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 β-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 µg ampicillin per ml, this point is reached when the culture is barely becoming turbid (about 10^7 cells per milliliter). The presence of 200 µg ampicillin per milliliter delays this point to a slightly higher cell density, but given the catalytic activity of β-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 β-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 β-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.

Precautions to Maximize Expression

Simple precautions are recommended to maximize retention of plasmid through the procedures for isolating, maintaining, and expressing target plasmids. Experiments at Novagen 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. A more detailed discussion of the potential advantages of kan^R compared to amp^R is presented in *Antibiotic Resistance*, p. 6.

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 µg/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.
3. As soon as the colony develops (usually overnight at 37°C), inoculate into 2 ml LB + 50 µg/ml carbenicillin and grow until OD_{600} = 0.5.

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

4. Mix 0.9 ml of culture with 0.1 ml of 80% glycerol in a cryovial and store in a –70°C freezer. If there is any question about the possible stability of the plasmid, perform the plasmid stability test (described in *Optimizing Expression* on p. 27) at the time of freezing to determine what fraction of the cells contain functional target plasmid.

The following induction protocol has been used successfully at Novagen 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 µg/ml carbenicillin. Grow the cells at 37°C until OD_{600} = 0.2–0.6.
2. Collect the cells by centrifugation (30 sec 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 β -lactamase.*

3. Collect the cells by centrifugation at 1000 × 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.

Rationale for Plasmid Stability Test

For cells that carry a plasmid but no source of T7 RNA polymerase, titering in the presence and absence of antibiotic determines the fraction of cells that have plasmid. When the target plasmid has a plain T7 promoter and is carried in a λDE3 lysogen, you can test the fraction of cells able to express the target gene. Do this by including 1 mM IPTG in the agar, which will prevent colony formation by any cell that has both the inducible gene for T7 RNA polymerase and a functional target plasmid. Note that this will not prevent growth of cells that lack plasmid or mutants that have lost the ability to express target DNA. In the presence of pLysS, IPTG also prevents colony formation except with certain vectors, including pET-3 and some that carry the T7*lac* promoter without a cloned insert. In the presence of pLysE, IPTG usually does not prevent colony formation unless the target gene product is toxic. Similarly, IPTG occasionally does not suppress colony formation with plasmids carrying the T7*lac* promoter and relatively innocuous target genes, especially in the presence of pLysS.

In practice, DE3 lysogens that carry a target plasmid are titered on four plates: (1) with the appropriate antibiotic, (2) with IPTG, (3) with both antibiotic and IPTG, or (4) with neither added to the plate. All viable cells will grow on the plate with no additive; only cells that retain plasmid

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will grow in the presence of antibiotic; only cells that have lost plasmid or mutants that have lost the ability to express target DNA will grow in the presence of IPTG (with the above exceptions); only mutants that retain plasmid but have lost the ability to express target DNA will grow in the presence of both antibiotic and IPTG (with the same exceptions). In a typical culture useful for producing target proteins, almost all cells will form colonies both on plates without additives and on plates containing only antibiotic; less than 2% of the cells will form a colony on plates containing only IPTG; and less than 0.01% will form a colony on plates containing both antibiotic and IPTG. With unstable target plasmids, the fraction of cells that have lost plasmid will be reflected by an increase in colonies on the IPTG plate and a decrease on the antibiotic plate. Mutants that retain plasmid but have lost the ability to express target DNA arise in some cases, but relatively infrequently.

If the plasmid is stable, cultures for expressing the target gene can be grown from the freezer stock without special precautions; even if the antibiotic in the fresh medium is destroyed or if the culture is incubated overnight at saturation, almost all of the cells will retain the target plasmid. However, if the target plasmid is unstable, cultures should be grown from a dilution of 10^4 or higher from the freezer stock and grown directly to the density used for expression. Because of the potential for loss of plasmid, determine the composition of the cells in the culture by plating immediately before induction. This simple test can be invaluable in interpreting any unusual properties of an induction and in making sure that effort is not wasted on processing cells that had suboptimal levels of expression.

F. Difficult Target Proteins

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 made in disappointingly small amounts, for reasons that are obvious in some cases and obscure in others. Here we briefly summarize some of the known or likely reasons for obtaining low levels of expression.

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.

Plasmids containing extremely toxic genes may be destabilized in λDE3 lysogens by cAMP mediated derepression of the *lacUV5* promoter, which may increase basal expression of T7 RNA polymerase. This derepression can be effectively delayed by including 1% glucose in the culture medium.

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 apparently 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.

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; Lathop 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, Arg. Processing ranged from 16%–97% when the remaining amino acids occupied this position.

Tobias et al. (1991) have determined the relationship between a protein's amino terminal amino acid and its stability in bacteria, i.e., the N-end rule. 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, and Tyr. In contrast, all other amino acids conferred half-lives of > 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 be targeted for rapid degradation. Therefore, when an *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.

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 (Preibisch et al., 1988; Halling and Smith, 1985). 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, GST•Tag and/or CBD•Tag N-terminal fusion and His•Tag C-terminal fusion. In this case, the full length target protein can be isolated by performing sequential affinity purification with immobilized T7•Tag antibody, S-protein (Kim and Raines, 1993, 1994), GST•Bind or CBIND resin and His•Bind resin.

Other Factors Influencing Expression Level

Besides toxicity and degradation, other factors that can influence the expression level of target proteins include the following:

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

   rbs *Nde* I/*Nco* I
   5'...AAGAAGGAGAUAUACAUAUG...3'
   5'...AAGAAGGAGAUAUACCAUGG...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.

2. Excessive rare codon usage in the target gene has also been implicated as a cause for low level expression (Zhang et al., 1991; Sorensen et al., 1989). The effect seems to be most severe when multiple rare codons occur near the amino terminus (Chen and Inouye, 1990). A number of studies have indicated that high usage of the Arg 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; Hua et al., 1994; Schenk et al., 1995; Zahn 1996, Calderone et al., 1996). It should be noted, however, that only a subset of codons characterized as rare in highly expressed *E. coli* genes have levels of cognate charged tRNAs that are low enough to present potential problems in translation elongation (Ikemura, 1985).

3. 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. A very convenient test is done using Novagen's Single Tube Protein System 3 (see p. 23).

Many target proteins seem to be made in equivalent amounts whether or not the Tϕ transcription terminator is present in the vector. In some cases, however, having Tϕ 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 amp^R pET vectors. Because Tϕ reduces the amount of this competing mRNA, it allows more target protein to be made. In the kanamycin-resistant pET vectors, where the kan gene and the target gene have opposite orientations, no competing mRNAs are known to be made along with the target mRNA.

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V. Detecting and Quantifying Target Proteins

Protein expression can be rapidly tested 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 commonly used as a more specific and sensitive method for identification and estimation of expression levels, and can be conveniently performed using fusion tag-specific reagents and kits 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, accurate and sensitive quantification of soluble S•Tag fusion proteins in crude extracts is made possible by the S•Tag Rapid Assay Kit.

The pET and pSCREEN plasmids offer a variety of vector-encoded fusion tags. These tags enable detection of target protein, measurement of expression levels and purification by several independent strategies. Specific protocols for Western blotting, immunoprecipitation, and rapid assay using Novagen's detection reagents and kits are available on request and also accompany the various products, as listed in the following table.

Detection/Assay Products for Fusion Tags

Product	Cat. No.	Technical Bulletin No.
S•Tag detection/assay		
S•Tag Rapid Assay Kit	69212-3	TB082
S•Tag AP Western Blot Kit	69213-3	TB082
S•Tag AP LumiBlot Kit	69099-3	TB164
S•Tag HRP LumiBlot Kit	69058-3	TB145
S-protein FITC Conjugate	69060-3	TB143
S-protein AP Conjugate	69598-3	TB097
S-protein HRP Conjugate	69047-3	TB136
T7•Tag detection		
T7•Tag Monoclonal Antibody	69522-3 (50 µg) 69522-4 (250 µg)	TB015
Biotinylated T7•Tag Antibody	69968-3	TB106
T7•Tag AP LumiBlot Kit	70237-3	TB212
T7•Tag HRP LumiBlot Kit	70238-3	TB213
T7•Tag Antibody AP Conjugate	69999-3	TB112
T7•Tag Antibody HRP Conjugate	69048-3	TB137
HSV•Tag detection		
HSV•Tag Monoclonal Antibody	69171-3 (40 µg) 69171-4 (200 µg)	TB067
GST•Tag detection/assay		
GST Assay Kit	70532-3	TB236
CBD•Tag detection		
CBD_{clos}•Tag Antibody	70119-3	
CBD_{cenA}•Tag Antibody	70157-3	
CBD_{cex}•Tag Antibody	70158-3	

VI. Purifying Target Proteins

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 pET vector construct, 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. 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. A general consideration regarding host strains involves the decision whether to use strains containing pLysS or pLysE. Whereas pET vectors that express target proteins in the cytoplasm may be successfully used with pLysS and pLysE strains, these strains 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).

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. Affinity resins and buffer kits for convenient purification using the T7•Tag, CBD•Tag, S•Tag, GST•Tag and His•Tag sequences are listed on the flow chart on p. 15 in *Getting Started*.

Prior to 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. The target protein may be found in any or all of the following fractions: soluble or insoluble cytoplasmic fractions, periplasm, or media. Depending on the intended application, preferential localization to inclusion bodies, media, or the periplasmic space can be advantageous for rapid purification by relatively simple procedures. Several methods for analyzing crude cell fractions are presented next.

A. Small Scale Analysis

The following small-scale protocol is designed to do the following:

- Verify that the target protein is produced upon induction
- Determine the cellular localization and estimate the percentage or relative amount of the target protein in each of the four fractions (based on normalized SDS-PAGE analysis)
- Verify the presence of detection tags in the target protein

To facilitate the gel and Western analysis, two worksheets are provided to record data and calculate normalized loading volumes for standard mini gels (pp. 39–40). If larger gels are used, the loading volumes should be scaled up accordingly. This formula relies on the generation of accurate harvest OD_{600} readings and the determination of concentration factors for the fractions generated. The concentration factor represents the volume of original culture used to produce the fraction divided by the final volume of the fraction. The loading volume of each sample will need to be calculated, because the actual concentration factor for a given sample may vary, e.g., media or periplasmic samples.

Growth and Induction

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 sterile loop of cells taken from a plate or glycerol stock.
2. Incubate at 37°C with shaking at 250 rpm to an OD_{600} of approximately 0.5. Then add the entire 3 ml culture to 100 ml LB media containing antibiotics.
3. Shake the 100 ml culture at the desired temperature until the OD_{600} is approximately 0.5–1.0 (e.g., 2–3 h in LB broth, 37°C). Monitor the OD_{600} during growth by removing aliquots aseptically.

4. Just prior to induction, split the 100 ml culture into 2 × 50 ml cultures. For plasmids having the T7*lac* promoter, add IPTG to 1 mM (500 µl of sterile 100 mM IPTG) to one of the 50 ml cultures. For "plain" T7 promoter vectors, use 200 µl IPTG for a final concentration of 0.4 mM. The other culture will serve as the uninduced control. Incubate 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 overnight).

Optical Density Analysis of the Induced Culture

1. After induction and just prior to harvest, shake well to ensure a homogeneous suspension and remove a 0.5–1 ml aliquot of the induced and uninduced cultures.
2. Determine the OD_{600} of the culture as accurately as possible. This is done by diluting 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 used for growth.
3. Record both the dilution factor and the OD_{600} reading on the attached worksheet (p. 39).

Total Cell Protein (TCP) Sample

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

Note: *As an alternative to this method, cells can be processed using BugBuster™ Protein Extraction Reagent as described in Section VI.B. BugBuster reagent lyses the bacteria and avoids the need to employ mechanical disruption (French Press, sonication) for cell lysis. If the BugBuster method is used, the soluble and insoluble fractions should be analyzed side by side by SDS-PAGE. The soluble fraction prepared with BugBuster reagent contains soluble periplasmic and soluble cytoplasmic proteins, and the insoluble fraction contains inclusion bodies, membrane-associated and other macromolecular complex-associated proteins.*

1. Prior to harvesting the cells, take a 1 ml sample of well-mixed culture and centrifuge at 10,000 × 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 µl of 1X phosphate-buffered saline (PBS) to yield a concentration factor of 10X (100 µl vs. starting volume of 1 ml culture).
3. Add 100 µl of 2X Sample Buffer (2X SB = 100 mM DTT, 2% SDS, 80 mM Tris-HCl, pH 6.8, 0.006% bromophenol blue, 15% glycerol) 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. Heat for 3 min at 70°C to denature the proteins and then store at –20°C until SDS-PAGE analysis.

Note: *An alternative procedure for the rapid generation of SDS-whole cell lysates has been reported. With this technique, viscosity due to chromosomal DNA is eliminated by precipitation with Mg^{2+} in the lysis buffer followed by centrifugation (Chen and Christen, 1997).*

Preparation of the four fractions is presented in the sequence: media–periplasm–soluble cytoplasm–insoluble cytoplasm because the material from one fraction can be used to prepare the next. The process can be customized to eliminate separate analysis of specific fractions, if desired. For example, analysis of the media fraction may be instructive mainly when performing prolonged inductions, when expecting protein export, or when leakage of the target protein 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.

Media Sample

1. Harvest 40 ml of the culture by centrifugation at 6,500 × g for 15 min at 4°C.
2. Carefully transfer a 1 ml sample of the 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 in the next section.

3. Concentrate the media sample by either TCA precipitation or spin filter concentration, as follows:

Trichloroacetic acid (TCA) precipitation

a) Add 100 µl (1/10 volume) of 100% TCA (w/v) to 1 ml of medium and vortex for 15 sec.

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 of 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 [Savant]). The presence of residual acetone will make resuspension more difficult.

f) Add 100 µl of 1X PBS (concentration factor = 10X) and 100 µl of 2X SB. Resuspend by vigorous vortex mixing or sonication.

g) Heat for 3 min at 70°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 SB using an amount of 2X SB equal in volume to that of the concentrated sample just removed from the device. Pool the 2X SB membrane rinse with the concentrated sample.

d) Heat for 3 min at 70°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 media fraction is expected to be very low unless substantial cell lysis has occurred.*

Periplasmic Fraction Sample–Osmotic Shock

When using vectors having *ompT*, *pelB*, CBD 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. Translocation across the cell membrane of *E. coli* is incompletely understood (reviewed by Wickner et al., 1991). 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 λDE3 lysogens. However, osmotic shock is not appropriate for use with host strains containing pLysS or pLysE because T7 lysozyme causes disruption of the inner membrane.

1. Resuspend the cell pellet (generated in step 2 of *Media Sample*, previous section) thoroughly in 30 ml of 30 mM Tris-HCl pH 8, 20% sucrose. Then add 60 µl 0.5 M EDTA, pH 8 (final concentration of 1 mM). Add a magnetic stirring bar and stir slowly at room temperature for 10 min.

2. Collect the cells by centrifugation at 10,000 × g at 4°C for 10 min. Remove all of the supernatant and discard.

3. Thoroughly resuspend the pellet in 30 ml of ice-cold 5 mM $MgSO_4$ 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 × g to pellet the shocked cells. Transfer a 1 ml sample from the supernatant (periplasmic fraction) to a microcentrifuge tube. Avoid removing any loose pellet with the supernatant.

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The excess supernatant (periplasmic fraction) may be removed and saved for activity assays, if desired. Save the cell pellet on ice for further processing to prepare the soluble and insoluble cytoplasmic fractions.

5. Concentrate the periplasmic fraction by TCA precipitation or spin filtration as described for the media sample in step 3 in the previous section (*Media Sample*). Again the desired concentration factor is 10X. Record the actual concentration factor on the worksheet (p. 40).
6. Add an equal volume of 2X SB and heat for 3 min at 70°C to denature the proteins. Store at –20°C until SDS-PAGE analysis.

Note: The success of this procedure can be monitored with the 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 by LaVallie et al. (1993) for extraction of *trxA* fusion proteins, which can be produced by pET-32 series vectors.

To extract trxA fusion proteins:

1. Resuspend induced cells in ice-cold 20% sucrose 2.5 mM EDTA, 20 mM Tris-HCl, pH 8.0, to a concentration of 5 OD_{550} units/ml and incubate on ice for 10 min.
2. Centrifuge at 15,000 × g for 30 seconds, decant the pellet, and resuspend in the same volume of ice-cold 2.5 mM EDTA, 20 mM Tris-HCl, pH 8.0. Incubate on ice for 10 min.
3. Centrifuge at 15,000 × g for 10 min. The supernatant is the osmotic shock fraction. Analyze supernatant and pellet by SDS-PAGE.

Soluble Cytoplasmic Fraction

As discussed in Section IV.C. *Optimizing Expression*, many target proteins are expressed in both soluble and insoluble forms. Crude soluble and insoluble fractions can be prepared by the following protocol. This protocol will work with any of the pET host strains. In principle, the lysozyme addition could be omitted with strains having pLysS or pLysE. For an alternative protocol that does not require mechanical breakage of the cells, please refer to the following section, *Preparation of Cell Extracts with BugBuster™ Protein Extraction Reagent.*

1. Completely resuspend the pellet from step 4 of the osmotic shock procedure above in 4 ml of cold 20 mM Tris-HCl pH 7.5 to yield a concentration factor of 10X (40 ml culture to 4 ml buffer volume).

Note: Some proteins may exhibit higher solubility when the cells are lysed in a buffer containing salt. If desired, NaCl up to 0.5 M 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. 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) *Lysozyme treatment plus sonication*. Add lysozyme to a final concentration of 100 µg/ml from a freshly prepared 10 mg/ml stock in water. Incubate at 30°C for 15 min. Mix 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 conditions for a given sonicator may be quickly determined by performing a time course analysis. Remove samples at various times during the sonication, centrifuge at 12,000 x g for 5 min, and then 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.

3. Take a 1.5 ml sample of the lysate and centrifuge at 14,000 × g for 10 min to separate the soluble and insoluble fractions. Transfer 100 µl of the soluble supernatant to a new tube. Remove and save the remaining supernatant for activity assays, as desired. Save the insoluble pellet fraction on ice for processing, as described in the next section.

4. Add 100 µl of 2X SB to the 100 µl soluble fraction sample. Heat for 3 min at 70°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

Insoluble Cytoplasmic Fraction

The insoluble fraction (inclusion bodies) can be further purified by repeated centrifugation and washing steps. However, the product will be contaminated at some level with other proteins and nucleic acids. In many cases, purified inclusion bodies are suitable for direct use as antigens for the preparation of antibodies against the target protein.

1. Wash the insoluble pellet by resuspending in 750 µl of 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 of 1% SDS with heating and vigorous mixing or sonication if necessary (resuspension in this volume maintains the concentration factor at 10X).
3. Take a 100 µl sample and add it to 100 µl of 2X SB. Heat for 3 min at 70°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

B. Preparation of Cell Extracts with BugBuster™ Protein Extraction Reagent

BugBuster Protein Extraction Reagent is formulated for the gentle disruption of the cell wall of *E. coli*, resulting in the liberation of soluble protein. It provides a simple, rapid, low cost alternative to mechanical methods such as French Press or sonication for releasing expressed target proteins in preparation for purification. The proprietary formulation utilizes a mixture of non-ionic detergents that is capable of cell wall perforation without denaturing soluble protein. Cells are harvested by centrifugation as usual, followed by suspension in BugBuster reagent. During a brief incubation soluble proteins are released. The extract is clarified by centrifugation, which removes cell debris and insoluble proteins. The clarified extract is ready to use and is fully compatible with any of the affinity chromatography resins offered by Novagen. The insoluble fraction can be further processed to yield purified inclusion bodies.

Soluble Fraction

Note: This fraction will consist of soluble protein present in both the periplasm and cytoplasm. If a separate periplasmic fraction is desired, follow the osmotic shock procedure given on pp. 36–37. The final pellet from the osmotic shock procedure can then be used in this protocol.

1. Harvest cells from liquid culture by centrifugation at 6,500 × g for 5 min. Decant and allow the pellet to drain, removing as much liquid as possible. Allow the cell pellet to equilibrate to room temperature.
2. Resuspend the cell pellet in BugBuster at room temperature, using 2 ml reagent for cells from a 50 ml culture (see notes below for other volumes). Vortex gently and avoid foaming.
3. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10 min at room temperature.
4. Remove insoluble cell debris by centrifugation at 16,000 × g for 20 min at 4°C. If desired, save the pellet for inclusion body purification as described below.
5. Transfer the supernatant to a fresh tube. The soluble extract can be loaded directly onto any Novagen protein purification resin (and numerous other systems). Maintain clarified extracts on ice for short term storage (< 1 day) or freeze at –20°C until needed (see notes below).

Inclusion Body Purification

1. Process the induced culture according to steps 1–4 above for the soluble protein fraction.
2. Resuspend the pellet from step 4 above in the same volume of BugBuster reagent that was used to suspend the cell pellet. Pipet the pellet material up and down and vortex to obtain an even suspension.
3. Add lysozyme to a final concentration of 200 µg/ml (use 1/50 volume of a freshly prepared 10 mg/ml stock in water). Vortex to mix and incubate at room temperature for 5 min.
4. Add 6 volumes of 1:10 diluted BugBuster reagent (in deionized water) to the suspension and vortex for 1 min.
5. Centrifuge the suspension at 16,000 × g for 15 min at 4°C to collect the inclusion bodies. Remove the supernatant with a pipet.

6. Resuspend the inclusion bodies in ½ the original culture volume of 1:10 diluted BugBuster, mix by vortexing, and centrifuge as in step 5. Repeat this wash step 2 more times.
7. Resuspend the final pellet of purified inclusion bodies in your buffer of choice. Inclusion bodies prepared in this manner are compatible with resuspension in 1X IB Solubilization Buffer provided in Novagen's Protein Refolding Kit (Cat. No. 70123-3).

Notes on the use of BugBuster reagent:

a) When processing cultures > 40 ml in scale, a volume of BugBuster corresponding to 1/25 of the original culture volume is recommended for resuspension of cell pellets. For culture volumes less than 40 ml, use up to 1/5 culture volume for resuspension (e.g., use 300 µl BugBuster for 1.5 ml cultures). There are no adverse effects to using larger volumes of BugBuster, as required, to simplify handling.

b) For small scale extractions (1.5 ml or less), centrifugation to remove cell debris and to collect inclusion bodies can be performed at top speed in a microcentrifuge (14,000–16,000 x g).

c) Extraction is most efficient when performed at room temperature. Protease inhibitors (e.g., Protease Inhibitor Cocktail Set II or III) can be added along with the BugBuster reagent if desired.

d) Extraction appears to be especially efficient with BL21 strains. More protein may be released if cells are frozen prior to processing.

e) Extracts should be stored at a temperature that is compatible with target protein activity; some target proteins may be inactivated by freeze-thaw cycles.

f) For SDS-PAGE and Western blot analysis (Section VI.C. below), a load volume of approximately 2.4 µl would give a normalized amount of protein, given a concentration factor of 25 and an OD_{600} of 3 at harvest, using a 15-well mini gel. When 1/5 culture volume of BugBuster is used, this would correspond to a load volume of approximately 12 µl (since the concentration factor is 5).

C. SDS-PAGE and Western Blot Analysis

Normalize the samples for loading based on the OD_{600} 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. No. 69149-3 or 69079-3) provide accurate size references for proteins between 10 kDa and 225 kDa on Coomassie blue stained gels.

Normalized SDS-PAGE Gel Loading

Worksheet 1: Determination of the culture OD_{600} at harvest.

	Dilution Factor (DF)	**OD_{600} of diluted sample**	**OD_{600} at harvest** ($DF \times OD_{600}$ of diluted sample)
Induced Culture			
Uninduced Culture			

Worksheet 2: Determination of the normalized volume of sample to load on a standard 10-well or 15-well mini SDS-PAGE gel.

				Volume to Load	
				15-well mini-gel	10-well mini-gel
	Sample conc. factor	OD_{600} at harvest	Z (conc. Factor × OD_{600})	180 µl ÷ Z	270 µl ÷ Z
Induced Samples					
TCP					
Media					
Periplasmic					
Soluble Cytoplasmic					
Insoluble Fraction					
Uninduced Samples					
TCP					
Periplasmic					
Media					
Soluble Cytoplasmic					
Insoluble Fraction					

The identity of the target protein can be determined by Western blotting with a target protein-specific antibody or detection based on vector-encoded fusion partners, such as the T7•Tag, CBD•Tag, GST•Tag and HSV•Tag antibodies or S-protein (for S•Tag fusions). For Western blots dilute the samples 1:25 to 1:50 in 1X SB and load along with Novagen's Perfect Protein Western Blot Markers (Cat. No. 69959-3). Because the markers carry the S•Tag sequence, they can be detected using the S-protein AP or HRP conjugates (McCormick et al., 1994). During blot processing, include either S-protein AP Conjugate or S-protein HRP Conjugate (Cat. Nos. 69598-3, 69047-3, respectively) to detect the markers (and target protein, if it possesses an S•Tag sequence).

D. Large Scale Induction and Fractionation

After the various parameters of growth, induction, and localization are established on a small scale, these can be applied to larger scale cultures for production of target proteins.

1. *Inoculation*: Inoculate 500 ml or more of media containing the appropriate antibiotics with a 3 ml starter culture grown to an $OD_{600} \leq 0.5$.
2. *Growth*: Incubate with shaking at 37°C or other desired temperature until the OD_{600} is approximately 0.5–1.0. If induction is to be performed at temperatures lower than 37°C, the cultures may be grown at 37°C until the OD_{600} is approximately 0.5 and then shifted to the desired temperature for at least 15 min prior to the addition of IPTG.
3. *Induction*: Induce expression by adding IPTG to a final concentration of 1 mM (T7*lac* promoter) or 0.4 mM ("plain" T7 promoter), and continue incubation at the optimized time and temperature.

Media Fraction

Harvest the induced culture by centrifugation at 6,500 × g for 15 min at 4°C. Transfer the supernatant (media fraction) to a new container and centrifuge at 12,000 × g for 15 min at 4°C to remove any residual bacteria. Recover the supernatant and store at 4°C.

Periplasmic Fraction

Note: This procedure is not appropriate for pLysS or pLysE host strains.

1. Harvest 500 ml of the induced culture by centrifugation at 6,500 × g for 15 min at 4°C. Resuspend the cell pellet thoroughly in 400 ml of 30 mM Tris-HCl pH 8.0, 20% sucrose. Add 0.8 ml of a 0.5 M pH 8.0 stock solution of EDTA (1 mM final concentration). Stir slowly at room temperature for 10 min.
2. Collect the cells by centrifugation at 10,000 × g at 4°C for 10 min. Remove all of the supernatant and discard. It is important to remove as much liquid as possible, especially when His•Bind chromatography will be used for purification, because residual EDTA may interfere with binding to the resin.
3. Thoroughly resuspend the pellet in 400 ml of ice-cold 5 mM $MgSO_4$ and stir the cell suspension slowly for 10 min on ice. Shocked cells should appear round instead of rod-shaped under the light microscope.
4. Centrifuge at 10,000 × g at 4°C for 10 min to pellet the cells and transfer the supernatant (periplasmic fraction) to a new tube and store at 4°C. Avoid removing any loose pellet with the supernatant.

Note: The following procedure uses mechanical disruption for cell lysis. Alternatively, soluble cell extracts and insoluble inclusion bodies can be prepared using BugBuster™ Protein Extraction Reagent, as described in Section VI.B.

Soluble Whole Cell Extract Fraction

1. Harvest the induced cells by centrifugation at 6,500 × g for 15 min at 4°C.
2. Decant and discard the media supernatant and thoroughly resuspend the cells in ice-cold buffer (e.g., 20 mM Tris-HCl pH 8.0 ± NaCl as determined by the small-scale optimization, or appropriate column binding buffer, such as 1X His•Bind Binding Buffer). Typically, 2–10 ml of buffer are used per gram of cells (wet weight). Resuspension is aided by pipetting up and down with a 10–25 ml pipette.
3. Cool the suspension on ice to 4°C to prevent heating during cell breakage. Lyse the cells by one of the following protocols. The two most commonly used methods for lysis of *E. coli* are sonication and high pressure (French Press) homogenization. Sonication is a more widely used technique, but two disadvantages are lower lysis efficiency and difficulty of scale-up beyond 50 g of cells (unless continuous flow sonication is used). Regardless of the lysis method used, keep samples cold to prevent proteolytic degradation of the target protein. Review articles on cell lysis provide detailed information on the various methods available (Tomme et al., 1994; Mukhopadhyay, 1997).
   a) *French Press*: Load 40 ml samples of suspended cells in a 50 ml capacity French pressure cell and break at 15,000 psi. Chill the pressure cell on ice between runs. One passage of the sample through the cell is usually sufficient to ensure adequate breakage. At this step, if the extract is viscous, the DNA will need to be sheared either by sonication or an additional pass through the French Press (after adding the protease inhibitors as described in step 4, if desired).
   b) *Lysozyme plus sonication*: Add lysozyme to a final concentration of 100 µg/ml from a freshly prepared 10 mg/ml stock in water. Incubate at 30°C for 15 min. Mix by swirling and sonicate on ice with an appropriate tip until cells are lysed and the solution is no longer viscous. It is important to keep the sample cold during sonication to avoid heat denaturation of proteins. The settings on p. 37 are general recommendations and may need to be adjusted depending on the energy output of a given sonicator. Consult sonicator manufacturer for recommended settings for your equipment.
4. *Optional*: Immediately following cell lysis, add protease inhibitors. To each 40 ml of homogenate, add 5 ml Protease Inhibitor Cocktail Set II with EDTA (Cat. No. 539132) or 1 ml Protease Inhibitor Cocktail Set III without EDTA (Cat. No. 539134). The cocktail without

EDTA is recommended when planning to use His•Bind Resin for purification of His•Tag fusion proteins by metal chelation chromatography.

5. Centrifuge the homogenate at 12,000 × g at 4°C for 30 min. Remove the soluble cell extract and save at 4°C for purification. The insoluble fraction (pellet) may be saved for inclusion body isolation and refolding, if necessary (see *Insoluble Whole Cell Extract Fraction*, next section).
6. Optional removal of nucleic acids prior to fusion protein purification: When processing large volumes of soluble cell extract (> 5 liters), it may be necessary to remove nucleic acids to avoid decreases in column flow rates and binding capacities. The bulk of nucleic acids can be removed by either polyethylenimine (PEI; Jendrisak and Burgess, 1975; Gegenheimer, 1990) or streptomycin sulfate precipitation. The amount of PEI necessary to precipitate non-target proteins and nucleic acids without precipitating the target proteins must be determined empirically.

   Polyethylenimine (PEI) precipitation

   a) Determine the amount of PEI necessary to clarify your lysate by performing a titration of PEI on a series of aliquots of your sample. Add 0–140 µl/ml of 10% PEI (Polymin P, Sigma; prepared in sterile deionized water) to each aliquot and vortex briefly.
   b) Centrifuge for 2 min at 14,000 × g.
   c) Load 5 µg samples of each supernatant in the series on an SDS-polyacrylamide gel and stain with Coomassie blue.
   d) The maximum level of PEI that does not precipitate your target protein can be applied to the entire protein sample. Add PEI slowly, with stirring, until the target PEI percentage is reached. Stir for an additional 10 min.
   e) Clarify by centrifugation at 10,000 × g for 10 min.
   f) Save the supernatant, which contains the target protein.

   Streptomycin sulfate precipitation

   a) Add 50 ml 30% (w/v) streptomycin sulfate (Calbiochem #5711) per liter of cell extract. Add the streptomycin sulfate slowly, with stirring. After addition, slowly stir the extract at 4°C overnight.
   b) Centrifuge the cell extract at 12,000 × g for 30 min at 4°C.
   c) Stir the clarified cell extract for an additional 24 h at 4°C to complete precipitation.
   d) Once again, centrifuge at 12,000 × g for 30 min at 4°C to produce a clarified cell extract.

Note: The streptomycin sulfate clarified extract must be used immediately for column purification to avoid the formation of residual precipitate that could impede flow rate.

Insoluble Whole Cell Extract Fraction–Isolation of Inclusion Bodies

Note: An alternative procedure for preparing inclusion bodies using BugBuster reagent is described in Section VI.B.

Inclusion bodies are isolated by centrifugation and washed twice with a buffer containing Triton X-100 to remove loosely associated contaminants. The purified inclusion bodies can then be washed once more with PBS and used “as is” for emulsification in appropriate adjuvant for immunization, or solubilized in preparation for refolding and/or further purification. Reagents for washing and solubilizing inclusion bodies, protein refolding, and a detailed protocol (TB234) are included in the Protein Refolding Kit (Cat. No. 70123-3).

1. Harvest the induced culture by centrifugation at 6,500 × g for 15 min at 4°C. Remove and discard the supernatant.
2. Thoroughly resuspend the cell pellet in 0.1 culture volume of 1X IB Wash Buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton X-100). Mixing may be necessary for full resuspension.
3. Lyse the cells sonication or French Press. See step 3 of *Soluble Whole Cell Extract Fraction* on p. 41 for details on lysis. Maintain the lysate at 0–4°C during the lysis procedure.
4. Optional addition of protease inhibitors. See step 4 of *Soluble Whole Cell Extract Fraction* for details.
5. Collect the pellet by centrifugation at 10,000 × g for 10 min.

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6. Remove the supernatant and thoroughly resuspend the pellet in 0.1 culture volume of 1X IB Wash Buffer.
7. Repeat centrifugation and save the pellet.
8. Again, thoroughly resuspend the pellet in 0.1 culture volume of 1X IB Wash Buffer. Transfer the suspension to a clean centrifuge tube with known tare weight.
9. Collect the inclusion bodies by centrifugation at 10,000 × g for 10 min. Decant the supernatant and remove the last traces of liquid by tapping the inverted tube on a paper towel.
10. Weigh the tube and subtract the tare weight to obtain the wet weight of the inclusion bodies. A typical wet weight for inclusion bodies is approximately 1–4 mg/ml of culture when the insoluble protein constitutes 10–40% of the total cell protein and the OD_{600} of the harvest is approximately 3.0.

Solubilization of Inclusion Bodies and Refolding Proteins

A variety of methods have been published describing refolding of insoluble proteins (Burgess, 1996; Frankel and Leinwand, 1996; Mukhopadhyay, 1997; Kurucz et al., 1995; Rudolph and Lilie, 1996; Marston and Hartley, 1990). 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 with refolding of the target protein.

Novagen's 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 TB234, available from Novagen.

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 (prior to refolding) is possible using His•Tag fusion proteins and His•Bind metal chelation chromatography (see TB054). In addition, S•Tag fusion proteins solubilized from inclusion bodies using 6 M urea can be purified under partially denaturing conditions by dilution to 2 M urea prior to chromatography on S-protein Agarose (see TB160 or TB087). Refolded fusion proteins can be affinity purified under native conditions using His•Tag, S•Tag, and other appropriate affinity tags (e.g. GST•Tag, CBD•Tag, T7•Tag).

Purification Products for Affinity Tags

Specific protocols for purification and protease cleavage using Novagen's reagents and kits are available on request and also accompany the various products, as listed in the following table.

Product	Cat. No.	Technical Bulletin No.
BugBuster reagent and kits		
BugBuster Protein Extraction Reagent	70584-3	TB245
BugBuster GST•Bind Purification Kit	70586-3	TB235
BugBuster His•Bind Purification Kit	70585-3	TB054
S•Tag purification		
S•Tag Thrombin Purification Kit	69232-3	TB087
S•Tag rEK Purification Kit	69065-3	TB160
S-protein Agarose	69704-3 (2 ml) 69704-4 (5 x 2 ml)	TB160
T7•Tag purification		
T7•Tag Affinity Purification Kit	69025-3	TB125
T7•Tag Antibody Agarose	69026-3	TB125
His•Tag purification		
His•Bind Resin	69670-3 (10 ml) 69670-4 (50 ml)	TB054
His•Bind Resin & Buffer Kit	70239-3	TB054
His•Bind Quick 300 Cartridges	70155-3 (pkg/10) 70155-4 (pkg/50)	TB054
His•Bind Quick 900 Cartridges	70156-3 (pkg/10) 70156-4 (pkg/50)	TB054
His•Bind Quick Columns	70159-3 (pkg/12) 70159-4 (pkg/60)	TB054
His•Bind Buffer Kit	69755-3	TB054
CBD•Tag purification		
CBIND 100 Resin	70120-3	TB189
CBIND 200 Resin	70121-3	TB189
CBIND 300 Cartridges	70124-3 (pkg/10) 70124-4 (pkg/50)	TB189
CBIND 900 Cartridges	70132-3 (pkg/10) 70132-4 (pkg/50)	TB189
CBIND ReadyRun Columns	70144-3 (pkg/12) 70144-4 (pkg/60)	TB189
CBIND Buffer Kit	70122-3	TB189
GST•Tag purification		
GST•Bind Resin	70541-3	TB235
GST•Bind Buffer Kit	70534-3	TB235
Protease cleavage		
Thrombin, Restriction Grade	69671-3	TB188
Biotinylated Thrombin	69672-3	TB188
Thrombin Cleavage Capture Kit	69022-3	TB188
Streptavidin Agarose	69203-3	TB188
Recombinant Enterokinase	69066-3	TB150
Enterokinase Cleavage Capture Kit	69067-3	TB150
EKapture™ Agarose	69068-3	TB150
Factor Xa, Restriction Grade	69036-3	TB205
Factor Xa Cleavage Capture Kit	69037-3	TB205
Xarrest™ Agarose	69038-3	TB205

VII. Induction Control: β-Galactosidase Recombinant

All pET vectors and systems include an induction control, which in many cases is a glycerol stock of an appropriate pET vector containing the *E. coli* β-galactosidase gene as an insert. These recombinants 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 on p. 14.

As well as providing controls for induction conditions, these strains can also be used to test protease cleavage of N-terminal fusion sequences with the appropriate enzyme. Because they express *E. coli* β-galactosidase as the target gene, enzymatic activity can be used to easily follow the protein through the purification and cleavage steps. Large amounts of the 116 kDa β-galactosidase protein accumulate in soluble form under standard induction conditions, and the protocols for purification on the appropriate affinity resin under native conditions may be followed to retain enzymatic activity. The enzyme could also be purified under denaturing conditions using urea or guanidine, but it would need to be renatured prior to an activity assay. Note that many of the host strains also produce native β-galactosidase; however, in most cases the amount of native enzyme is insignificant compared with the amount expressed from induced pET recombinants.

Treatment of the purified β-galactosidase fusion protein with the appropriate site-specific protease produces a large product (116 kDa β-gal enzyme) plus a small product (the fusion tag). Because gel analysis of cleavage is not always possible due to the small size of some of the fusion tags, an alternative assay can be used in which a sample of cleaved protein is bound batchwise to the appropriate affinity resin. After centrifugation to remove the resin, the unbound supernatant is analyzed by SDS-PAGE. If cleavage was complete, all of the target protein should be found in the unbound supernatant. This approach could also be modified to follow the cleavage of enzymes (or any assayable target protein).

Note that functional β-galactosidase is a tetrameric protein and the formation of tetramers is required for activity. Both cleaved and uncleaved peptide chains can associate to give functional molecules. Therefore, the amount of unbound protein under represents the degree to which cleavage has occurred. Thus, the activity assay is only semi-quantitative as an estimate of protease digestion until cleavage is complete.

β-Galactosidase Assay

Prepare 2X Reaction Buffer (200 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 1.33 mg/ml o-nitrophenyl-β-galactopyranoside [Calbiochem #48712]) and store frozen at –20°C in aliquots. Prepare Stop Buffer (1 M sodium carbonate) to terminate the reaction.

1. For an assay, thaw 2X Reaction Buffer and add 2-mercaptoethanol to 100 mM.
2. To a sample containing β-galactosidase, add water to a total of 75 µl, then add 75 µl of 2X Reaction Buffer.
3. Mix and incubate at room temperature for 5–10 min.
4. Terminate the reaction by adding 250 µl of Stop Buffer, and read the absorbance at 420 nm.

Because this assay is used to follow relative enzyme activity, the exact time and temperature used are not critical. To increase the sensitivity, the incubation time can be increased and/or the temperature raised to 37°C. Typical results from a His•Bind affinity purification of β-galactosidase are presented in the following table:

Fraction	Sample (µl)	A_{420}	Vol (ml)	% Activity
crude	0.02	0.28	3.25	100
flow-through	1	0.21	3.25	0.2
Binding Buffer wash	10	0.04	10.0	0.1
Wash Buffer	10	0.09	6.0	0.2
Elute Buffer	0.05	0.32	6.0	85.2
Strip Buffer	10	0.04	5.0	0.1

VIII. Acknowledgments

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X. Index

XI. Academic and 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: (516) 344-3312.
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:

   E. coli BL21(DE3)
   E. coli BL21(DE3)pLysS
   E. coli BL21(DE3)pLysE
   E. coli BL26(DE3)pLysE
   E. coli BL21*trxB*(DE3)
   E. coli BL21*trxB*(DE3)pLysS
   E. coli BLR(DE3)
   E. coli BLR(DE3)pLysS
   E. coli AD494(DE3)
   E. coli AD494(DE3)pLysS
   E. coli HMS174(DE3)
   E. coli HMS174(DE3)pLysS
   E. coli HMS174(DE3)pLysE
   E. coli B834(DE3)
   E. coli B834(DE3)pLysS
   E. coli NovaBlue(DE3)
   Bacteriophage λCE6
   Bacteriophage λDE3

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.