pET System Manual

This second printing of the 10th edition of the pET Manual was published May, 2003. Novagen is continually expanding and upgrading the pET System. Please check the Novagen website, www.novagen.com, for updated pET System Manual information.

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* patent pending

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All Novagen products are sold for research use only

I. About the System

A. Description

The pET System is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after induction. Although this system is extremely powerful, it is also possible to attenuate expression levels simply by lowering the concentration of inducer. Decreasing the expression level may enhance the soluble yield of some target proteins. Another important benefit of this system is its ability to maintain target genes transcriptionally silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell (see Section I. F. for details). Once established in a non-expression host, target protein expression may be initiated either by infecting the host with λCE6, a phage that carries the T7 RNA polymerase gene under the control of the λ p_L and p_I promoters, or by transferring the plasmid into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control. In the second case, expression is induced by the addition of IPTG to the bacterial culture. Although in some cases (e.g., with innocuous target proteins) it may be possible to clone directly into expression hosts, this approach is not recommended as a general strategy. Two types of T7 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 20.

All of the pET vectors and companion products are available as kits designed for convenient cloning, expression, detection, and purification of target proteins. The pET Expression Systems provide the plasmids and host strains. The background information 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 page 64.

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

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

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

- 0.2 ml aliquot each of NovaBlue, BL21(DE3) and BL21(DE3)pLysS 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 the Origami™ series hosts strains in addition to the BL21 series hosts.

Separate components and related products: see the Novagen Catalog or Novagen website (www.novagen.com) for a complete listing of pET vector DNA, systems and competent cells.

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D. Choosing a pET Vector

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 vectors developed at Novagen offer enhanced features to permit easier cloning, detection, and purification of target proteins. There are two general categories of vectors available: transcription vectors and translation vectors.

- Transcription vectors are designed for expression of target genes that already carry their own prokaryotic ribosome binding site and AUG start codon. There are only three transcription vectors: pET-21(+), pET-24(+) and pET-23(+).
- Translation vectors contain the highly efficient ribosome binding site from the phage T7 major capsid protein and are used for the expression of target genes without their own ribosomal binding site. Review the *pET Vector Characteristic Table* for the entire selection of translation vectors (page 8).

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

Primary considerations

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

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

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

Any known information available about the target protein may help determine the choice of vector. For example, some proteins require no extraneous sequence on one or both termini for activity. Most pET vectors enable cloning of unfused sequences; however, expression levels may be affected if a particular translation initiation sequence is not efficiently utilized in *E. coli*. In these cases, an alternative is to construct a fusion protein with efficiently expressed amino terminal sequences (indicated on the *pET Vector Characteristics Table*, page 8, with an N) and then remove the fusion partner following purification by digestion with a site-specific protease. Ligation-independent cloning (LIC) is especially useful for this strategy, because the cloning procedure enables the removal of all amino terminal vector-encoded sequences with either enterokinase or Factor Xa (as indicated in the *pET Vector Characteristics Table*, page 8).

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

Solubility and cellular localization

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

The choice of vector and expression host can significantly increase the activity and amount of target protein present in the soluble fraction. A vector can enhance solubility and/or folding in one of three ways: 1) provide for fusion to a polypeptide that itself is highly soluble [e.g. glutathione-S-transferase (GST), thioredoxin (Trx), N utilization substance A (NusA)], 2) provide for fusion to an enzyme that catalyzes disulfide bond formation (e.g. thioredoxin, DsbA, DsbC), or 3) provide a signal sequence for translocation into the periplasmic space. When using vectors designed for cytoplasmic expression, folding can be improved in hosts that are permissive for the formation of disulfide bonds in the cytoplasm (e.g. *trxB* and *gor* mutations, see page 15).

An alternative strategy to obtain active, soluble proteins is to use vectors that enable export into the periplasm, which is more favorable environment for folding and disulfide bond formation. For this purpose vectors carrying signal peptides are used. DsbA and DsbC are periplasmic enzymes that catalyze the formation and isomerization of disulfide bonds in pET-39b(+) and pET-40b(+) respectively. Note that other pET vectors that carry signal sequences without the additional DsbA or DsbC coding regions are also available (see chart on page 8).

In many cases target protein accumulates as insoluble inactive aggregates known as inclusion bodies. Inclusion bodies can be an advantage for purification because 1) they are easily isolated by centrifugation to yield highly concentrated and relatively pure protein, and 2) inclusion body formation protects the protein from proteolytic attack. In addition, toxic proteins may not inhibit cell growth when present in inactive form as inclusion bodies.

Some purification strategies optimize production of insoluble inclusion bodies in the cytoplasm. Inclusion bodies are extracted and solubilized; then the target protein is refolded *in vitro*. This procedure usually produces the highest yields of initial protein mass and protects against proteolytic degradation in the host cell. However, the efficiency of refolding into active protein varies significantly with the individual protein and can be quite low. Therefore, this approach is often used for producing antigens or in other applications for which proper folding is not required. pET-17xb expresses the full 220 aa T7 gene *10* protein as an N-terminal fusion and typically produces inclusion bodies. Also, pET-31b(+) is specifically designed for the generation of insoluble fusion proteins and provides a powerful method for the production of small proteins and peptides.

Fusion tags

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

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

The His•Tag sequence is very useful as a fusion partner for purification of proteins in general. It is especially useful for those proteins initially expressed as inclusion bodies, because affinity purification can be accomplished under totally denaturing conditions that solubilize the protein.

The CBD•Tag™ sequences are also generally useful for low cost affinity purification. They are also uniquely suited to refolding protocols [especially pET-34b(+) and 35b(+), which contain the

CBD_{clos}•Tag sequence]; because only properly folded CBDs bind to the cellulose matrix, the CBinD™ affinity purification step can remove improperly folded molecules from the preparation. While many of the tags can be used to immobilize target proteins, the CBD•Tag sequences are ideally suited for this purpose due to the inherent low non-specific binding and biocompatibility of the cellulose matrix.

The Nus•Tag™, Trx•Tag™ and GST•Tag™ sequences have been reported to enhance the solubility of their fusion partners. The ampicillin-resistant Nus•Tag and Trx•Tag vectors are compatible with Origami™, Origami B, and Rosetta-gami™ host strains, which facilitate disulfide bond formation in the cytoplasm (see page 15).

The various fusion tags available and corresponding vectors are listed in the following table. A number of pET vectors carry several of the fusion tags in tandem as 5' fusion partners (see page 8). In addition, many vectors enable expression of fusion proteins carrying a different peptide tag on each end. Using vectors with protease cleavage sites (thrombin, Factor Xa, enterokinase) between the 5' tag and the target sequence enables optional removal of one or more tags following purification. It should be noted that the expression of desired C-terminal fusions requires (1) the lack of a stop codon in the insert and (2) the proper reading frame at the cloning junction.

Fusion Tags Available for pET Constructs

Tag	N/C Terminal or Internal (I)	Size (aa)	Basis for Detection and/or Purification	Applications	pET Vector Series
T7•Tag®	N, I	11 or 260	monoclonal antibody	AP, IF, IP, WB	3, 5, 9, 11, 17 17x, 21, 23, 24, 28, 33
S•Tag™	N, I	15	S-protein (104aa) affinity	AP, QA, WB	29, 30, 32, 34–37, 39–44
His•Tag®	N, C, I	6, 8, or 10	metal chelation chromatography (native or denaturing) monoclonal antibody	AP, IF, WB	14–16, 19–44
HSV•Tag®	C	11	monoclonal antibody	IF, WB	25, 27, 43.1, 44
pelB*/*ompT	N	20/22	potential periplasmic localization	PE	12, 20, 22, 25, 26, 27
KSI	N	125	highly expressed hydrophobic domain	PP	31
Trx•Tag™	N	109	thioredoxin promotes disulfide bond formation – especially in *trxB* and *trxB*/*gor* hosts	DB, SP	32
PKA site	I	5	protein kinase A recognition site	PS	33
CBD_{clos}•Tag	N	156	polyclonal antibody, cellulose binding domain	IP, AP, WB	34, 35
CBD_{cenA}•Tag	N	114	polyclonal antibody, cellulose binding domain, periplasm/media	IP, AP, PE, WB	36, 37
CBD_{cex}•Tag	C	107	polyclonal antibody, cellulose binding domain, periplasm/media	IP. AP, PE, WB	38
Dsb•Tag™	N	208 (DsbA) 236 (DsbC)	potential periplasmic localization	DB, DI, PE, SP	39, 40
GST•Tag™	N	220	monoclonal antibody, enzymatic activity, glutathione affinity	AP, IF, IP, QA, WB	41, 42
Nus•Tag™	N, I	495	promotes cytoplasmic solubility-monoclonal antibody	SP, WB	43.1, 44

AP = affinity purification
DB = disulfide bond
DI = disulfide bond isomerization
IF = immunofluorescence
IP = immunoprecipitation
PE = protein export
PP = small protein/peptide production
PS = *in vitro* phosphorylation
QA = quantitative assay
SP = soluble protein
WB = Western blotting

pET Vector Characteristics Table

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.

Vector	amp^R	kan^R	T7	T7*lac*	His•Tag®	T7•Tag®^{11}	T7•Tag^{260}	S•Tag™	Trx•Tag™	CBD•Tag™	KSI	HSV•Tag®	PKA	Dsb•Tag™	GST•Tag™	Nus•Tag™	protease	signal seq.
pET-3a-d	●		●			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							I				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-41 Ek/LIC		●		●	I,C			I							N		T,E	
pET-42a-c(+)		●		●	I,C			I							N		T,X	
pET-43.1a-c(+)	●			●	I,C			I				C				N	T,E	
pET-43.1 Ek/LIC	●			●	I,C			I				C				N	T,E	
pET-44a-c(+)	●			●	N,I,C			I				C				I	T,E	

Vector amp^R kan^R T7 T7*lac* His•Tag T7•Tag^{11} T7•Tag^{260} S•Tag Trx•Tag CBD•Tag KSI HSV•Tag PKA Dsb•Tag GST•Tag Nus•Tag protease signal seq.

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

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E. pET Vector Cloning Strategies

Many strategies can be used for subcloning a protein-coding region of DNA into a pET vector for expression. Directional cloning can be accomplished with unique restriction sites in the multiple cloning region or through ligation-independent cloning (LIC) sites. The LIC method does not require restriction digestion or ligation and the LIC-prepared insert can be quickly cloned into multiple LIC vectors for high throughput cloning. For maps of all of the pET vectors please visit our website at www.novagen.com.

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

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

Produce native proteins without fusions

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

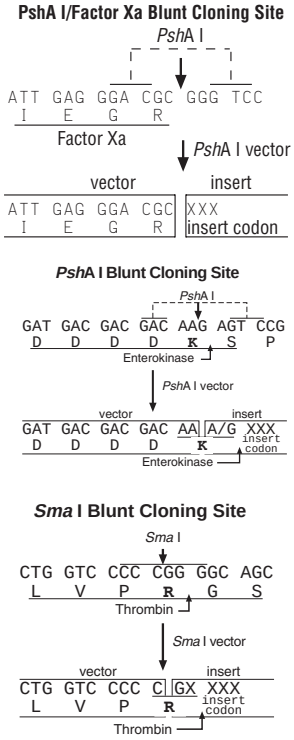
In many pET vectors the ATG triplet within the *Nco* I site (CCATGG) encodes the N-terminal methionine AUG start codon in the T7 RNA polymerase transcripts. Target genes or PCR engineered inserts that contain either *Nco* I sites or sites that generate compatible overhangs [*Bsp*H I (TCATGA), *Bsp*LU11 I (ACATGT), and subsets of *Afl* III (ACRYGT) and *Sty* I (CCWWGG)] at the beginning of their ORF can be cloned into the *Nco* I site. Note, however, that utilization of these restriction sites can be complicated if the target gene encodes multiple internal sites. In addition, each of these restriction sites dictates the first nucleotide of the next triplet codon, which may prevent the generation of native target protein. In such cases, it may be possible to employ restriction enzymes that cleave "downstream" of their recognition site, to allow the generation of native target protein (see table below).

Enzyme (isoschizomers)	Recognition and cleavage site	Overhangs generated	
Bbs I (*Bpi* I, *Bpu*A I)	5′-GAAGAC(N)$_2$ –3′	GAAGACNN	NNNNN
	3′-CTTCTG(N)$_6$ -5′	CTTCTGNNNNNN	N
Bsa I (*Eco*31 I)	5′-GGTCTC(N)$_1$ -3′	GGTCTCN	NNNNN
	3′-CCAGAG(N)$_6$ -5′	CCAGAGNNNNN	N
*Bsm*B I (*Esp*3 I)	5′-CGTCTC(N)$_1$ -3′	CGTCTCN	NNNNN
	3′-GCAGAG(N)$_5$ -5′	GCAGAGNNNNN	N
*Bsp*M I	5′-ACCTGC(N)$_4$ –3′	ACCTGCNNNN	NNNNN
	3′-TGGACG(N)$_8$ -5′	TGGACGNNNNNNNN	N

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

Produce native proteins without fusions after protease cleavage

The GST•Tag™ [pET-41a-c(+), 42a-c(+)] and Nus•Tag™ [pET-43.1a-c(+), pET-44a-c(+)] vectors contain *Psh*A I or *Sma* I restriction sites within sequences encoding Factor Xa, enterokinase or thrombin cleavage sites. Utilization of these blunt cutting restriction enzymes (as shown below) allows all vector-encoded protein sequences to be removed from the resulting fusion proteins by enterokinase, Factor Xa or thrombin digestion. The efficiency of thrombin cleavage can be affected by the nature of the amino acids immediately following the cleavage site. Optimal thrombin cleavage is obtained when the first two to three insert-defined amino acids are apolar and non-acidic (Chang, 1985; Le Bonniec, 1991; Le Bonniec, 1996).



Ligation-independent cloning

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 prepared pET vectors have non-complementary 12–15 base single-stranded overhangs that anneal to complementary single stranded overhangs on the target insert. Primers amplifying the target insert require the addition of 5' extensions to create the complementary sequence to the prepared LIC vector. The 3'→ 5' exonuclease activity of T4 DNA polymerase produces the single stranded overhang on the insert during a short incubation. Cloning is directional, and is very fast and efficient because only the desired product is formed by annealing the prepared plasmid and insert. An additional feature of the pET LIC vectors is the removal of all vector-encoded amino acids with the site specific proteases enterokinase or Factor Xa. See Technical Bulletins 163 and 205 for additional LIC cloning strategy information.

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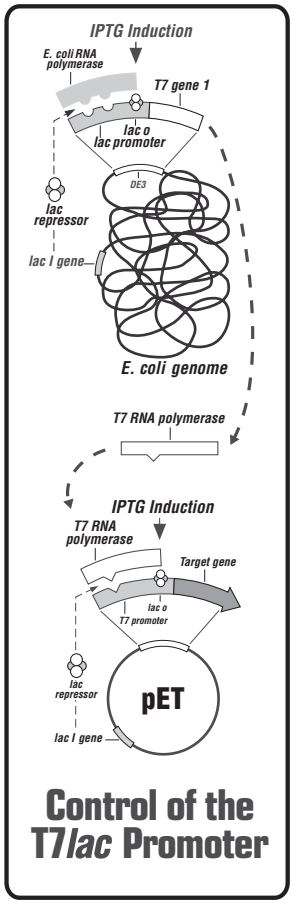
F. Regulating Protein Expression in the pET System

Even in the absence of IPTG, there is some expression of T7 RNA polymerase from the *lacUV5* promoter in λDE3 lysogens and therefore basal expression of the target protein. Any recombinant protein expressed in *E. coli* may interfere in normal functioning of the cell and therefore may be "toxic" to the bacteria. The degree of toxicity will vary from protein to protein. If target gene products are sufficiently toxic to *E. coli*, this basal level can be enough to prevent vigorous growth and the establishment of plasmids in λDE3 lysogens. The pET System is a powerful protein expression tool because you can tightly control protein expression with the T7/T7*lac* promoter, pLysS or pLysE hosts, and addition of glucose to the media based on the characteristics of your target protein. It should be noted that it is possible to over-regulate the system with the result being low protein expression levels. Therefore it is important to both understand the following tools and empirically determine what combination is best suited for each protein of interest.



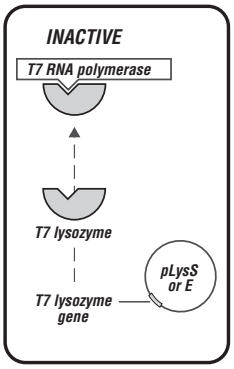
Control of the T7*lac* Promoter

The T7*lac* promoter

One approach to control basal expression is to use vectors that contain what is termed a T7*lac* promoter (Studier et al., 1990; Dubendorff and Studier, 1991; see table on page 8). 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, 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). Note that in combination with pLysS and pLysE hosts, expression can be over-regulated (see *Vector and host combinations affect expression levels* on page 12).

pLysS and pLysE hosts

Another way of providing additional stability to target genes is to express them in host strains containing a compatible chloramphenicol-resistant plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase (Moffatt 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 (Zhang and Studier, 1997; Huang et al., 1999). 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. Note that expression of lysozyme from pLysS hosts is also dependent on culture conditions. Because the upstream chloramphenicol acetyl transferase (CAT) antibiotic resistance gene is regulated by a catabolite repression sensitive promoter, growing pLysS host strains to stationary phase in the absence of glucose can lead to high cAMP and higher CAT promoter activity. The higher CAT promoter activity may be the cause of elevated lysozyme levels observed in cultures grown to stationary phase (Novy and Morris, 2001). When produced from the cloned gene, relatively high levels of T7 lysozyme can be tolerated by *E. coli* (i.e. no cell lysis), apparently because the protein is unable to pass through the inner membrane to reach the peptidoglycan layer.



Neither lysozyme plasmid interferes with transformation of cells that contain it; pLysS has little effect on growth rate but pLysE causes a significant decrease in the growth rate of cells that carry it. The higher level of lysozyme provided by pLysE can substantially increase the lag time and reduce the maximum level of expression of target genes upon induction of T7 RNA polymerase. This damping effect on expression is sufficient that cells containing a target gene whose product is relatively innocuous can continue to grow indefinitely in the presence of IPTG, a property that may be useful in some circumstances. The presence of either pLysS or pLysE

increases the tolerance of 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. PopCulture® and BugBuster®Protein Extraction Reagents release substantially more protein when used alone with hosts containing the pLysS and pLysE plasmids. This property can make it advantageous to carry pLysS in the cell even when it is not required for stabilizing the target plasmid. Note that the pLysS or pLysE plasmids are not recommended for use with constructs containing a signal sequence if isolation of the periplasmic fraction is desired (due to the breakdown of the cell membrane by the T7 lysozyme produced in those hosts).

Vector and host combinations affect expression levels

In practice, it is usually worthwhile to test several different vector/host combinations to obtain the best possible yield of protein in its desired form. When the "plain" T7 promoter is used, the low level of lysozyme provided by pLysS has little effect on expression of target genes following induction of T7 RNA polymerase, except for a short lag in the appearance of target gene products. Apparently, more T7 RNA polymerase is induced than can be inhibited by the small amount of lysozyme. (The level of lysozyme might be expected to increase somewhat upon induction, since T7 RNA polymerase should be able to transcribe completely around the pLysS plasmid from the 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. For an example illustrating differences in the expression of two target proteins with various combinations of T7/T7*lac* promoter and pLysS and pLysE hosts review Mierendorf et al., 1994.

Media containing glucose

As first described by Grossman et al. (1998), low basal expression levels in the pET system can be maintained by supplementing the medium with glucose. As cultures reach stationary phase, any available glucose is consumed first and an alternative carbon source such as glycerol is then utilized. Metabolism of the alternate carbon source causes cyclic AMP (cAMP) levels to increase, stimulating transcription from the *lacUV5* promoter and subsequent expression of T7 RNA polymerase in λDE3 lysogens. In contrast to the wild type *lac* promoter, the *lacUV5* promoter is not as sensitive to cAMP stimulation (Eron and Block, 1971; Fried and Crothers, 1984). However, it has been demonstrated that sufficient stimulation occurs to elevate T7 RNA polymerase levels, and consequently, T7 promoter regulated target gene expression (Kelley, 1995; Grossman et al., 1998; Pan and Malcom, 2000; Novy and Morris, 2001). A significant decrease in basal transcription from the *lacUV5* promoter is observed when standard medium is supplemented with glucose in cultures grown to stationary phase (Grossman et al., 1998; Pan and Malcom, 2000; Novy and Morris, 2001).

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

Note that addition of glucose is neither necessary nor recommended during the cloning steps in non-expression hosts. Although growing cultures to stationary phase is not recommended,

glucose provides another method to maintain the lowest basal levels of target protein in λDE3 lysogenic expression hosts used in the pET System and prevents overproduction of T7 lysozyme.

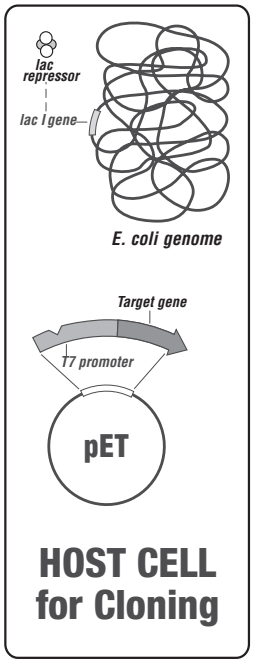
pLacI hosts

The specialized (DE3)pLacI based expression hosts are only intended for use with the high copy number pETBlue™ and pTriEx™ (1.1, 2, 3, and 4) series of vectors. These hosts supply *lac* repressor from the compatible pLacI plasmid to ensure stringent repression in the uninduced state. Host-provided *lac* repressor is required in pETBlue and pTriEx expression hosts because these plasmids do not contain the *lac* repressor gene. Refer to the pETBlue System Manual (Technical Bulletin 249) or the pTriEx System Manual (Technical Bulletin 250) for further details on use of pLacI hosts.

pETcoco™ System

Another approach to minimize basal expression in λDE3 lysogens is made possible by using the pETcoco vectors. These vectors are normally maintained as a single copy per cell, in contrast to the pET vectors which exist as 20–50 copies per cell. When present as a single copy, target genes become extremely stable, both due to minimal opportunity for recombination or gene rearrangement, and reduction in basal transcription levels to about 1/40 of pET vectors. Induction of protein expression with pETcoco recombinants is performed by inducing with IPTG and induced expression levels are similar to the pET vectors. For details see Sektas and Szybalski, 2002 and Technical Bulletin 333.

G. Hosts for Cloning

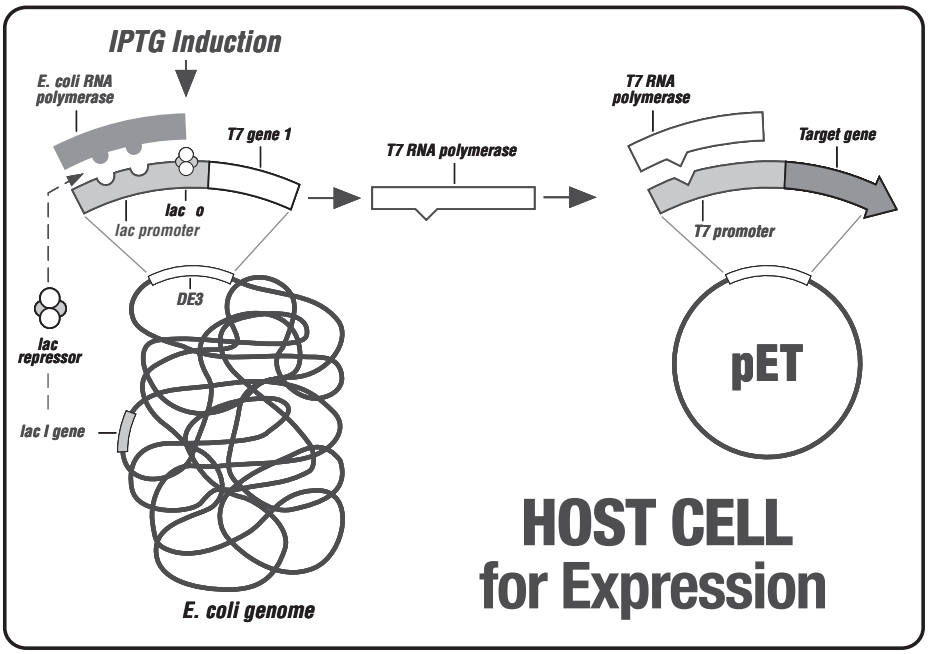


As described previously, a powerful feature of the pET system is the ability to clone target genes under conditions of extremely low transcriptional activity, that is, in the absence of a source of T7 RNA polymerase. Background expression is minimal in the absence of T7 RNA polymerase because the host 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, this approach is not recommended as a general strategy. Even low levels of basal expression can cause difficulties in growth and plasmid instability in these expression hosts due to transcription from the T7 promoter in the pET plasmids.

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 have high transformation efficiencies and good plasmid yields. NovaBlue has the additional advantage of having a selectable F factor that allows helper phage infection and therefore the production of single stranded plasmid DNA for mutagenesis purposes (appropriate only for plasmids carrying the f1 origin of replication). Note that there are no blue/white screening capabilities in the pET System because the pET vectors do not encode the *lacZ* α-peptide. If blue/white screening in a T7 expression vector is required, the pETBlue plasmids provide this option in combination with NovaBlue (see Technical Bulletin 249). If desired, expression can be induced in the NovaBlue host or other non-DE3 hosts by infection with the bacteriophage λCE6. See *Bacteriophage CE6* (page 18) for details.

H. Hosts for Expression

For protein production, a recombinant plasmid is transferred to an *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase (T7 gene *1*, see example below). These hosts are lysogens of bacteriophage DE3, a lambda derivative that has the immunity region of phage *21* and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase (Studier and Moffatt, 1986; Novy and Morris, 2001). 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 production, which in turn transcribes the target DNA in the plasmid. DE3 lysogen strains may be chosen for protease deficiency, amino acid auxotrophy, solubility enhancement, rare codon supplementation or other features. In addition, Novagen offers the λDE3 Lysogenization Kit, which allows the conversion of other *E. coli* strains to DE3 lysogens. It should be noted that several popular commercial cloning vectors carry T7 promoters and separate *lac* operator/promoter elements for blue/white screening of recombinants. While in principle these vectors could be used with the pET expression hosts, these vectors are inappropriate for this purpose. The multiple copies of the *lac* operator on these plasmids will titrate *lac* repressor and partially induce the gene for T7 RNA polymerase in the pET host, which is also controlled by *lac* repressor. As a result, basal T7 RNA polymerase activity becomes high enough that many target genes cannot be stably maintained. These elements are properly balanced in the pETBlue™ System.



Protease deficiency

All of the B strains, B834, BL21, BLR, Origami™ B, Rosetta™, and Tuner™ are deficient in the *lon* protease and lack 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 these strains than in host strains containing these proteases. BL21(DE3) is the most widely used host for target gene expression. BLR(DE3) is a *recA*$^-$ derivative of BL21 constructed by A. Roca, University of Wisconsin, and may stabilize some target genes containing repetitive sequences. The Origami B, Rosetta and Tuner strains are described in detail in the following sections.

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Adjustable expression levels throughout all cells in a culture

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

Disulfide bond formation and solubility enhancement

Many proteins require the formation of stable disulfide bonds to fold properly into a native conformation. Without disulfide bonds, these proteins may be degraded or accumulate as inclusion bodies. A limitation of the production of properly folded proteins in *E. coli* has been the relatively high reducing potential of the cytoplasmic compartment; disulfide bonds are usually formed only upon export into the periplasmic space. Bacterial strains with glutathione reductase (*gor*) and/or thioredoxin reductase (*trxB*) mutations (AD494, BL21*trxB*, Origami, Origami B, Rosetta-gami™) enhance the formation of disulfide bonds in the *E. coli* cytoplasm (Prinz et al., 1997; Aslund et al., 1999). AD494(DE3) and BL21*trxB*(DE3) have the *trxB* mutation while Origami(DE3), Origami B(DE3) and Rosetta-gami(DE3) strains carry the *trxB* mutation and the *gor* mutation. The *trxB* and *gor* mutant strains have the potential to enhance disulfide bond formation and ultimately solubility and activity to a greater degree than the *trxB* only mutants (Bessette et al., 1999). Studies have shown that expression in Orgami(DE3) yielded 10-fold more active protein than in another host even though overall expression levels were similar (Prinz et al., 1997). Note that the *trxB* mutation is maintained by kanamycin selection. Therefore these strains are not appropriate for expression of target genes cloned in kanamycin resistant plasmids. Also, note that Origami and Rosetta-gami are K-12 strains, while Origami B is a B strain deficient in the *ompT* and *lon* proteases, and carries the *lacY1* mutation.

Rare codon supplementation

Most amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons. In each cell, the tRNA population closely reflects the codon bias of the mRNA population. When the mRNA of heterologous target genes is over expressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. Insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshifting and amino acid misincorporation. The Rosetta™ strains are designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* (Brinkmann et al., 1989; Seidel et al., 1992; Kane, 1995; Kurland and Gallant, 1996). Expression of such proteins can be dramatically increased when the level of rare tRNA is increased within the host (Brinkmann et al., 1989; Seidel et al., 1992; Rosenberg et al., 1993; Del Tito et al., 1995). Rosetta strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC and GGA on a compatible chloramphenicol-resistant plasmid. These strains provide enhanced expression of target genes otherwise limited by the codon usage of *E. coli* (Novy et al., 2001). The tRNA genes are driven by their native promoters. In the pLysS and pLacI Rosetta strains, the rare tRNA genes are present on the same plasmids that carry the T7 lysozyme and *lac* repressor genes, respectively. The Rosetta series is derived from the BL21 *lacY1* mutant Tuner™ strain. RosettaBlue™ and Rosetta-gami™ strains are derived from and contain the features of the corresponding NovaBlue and Origami™ strains.

Selenomethionine labeling

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

pET system host strain characteristics table

This table lists the genotypes of strains commonly used for cloning and expression with the pET System; they are available from Novagen as glycerol stocks or competent cells ready for transformation. The catalog numbers for host strain glycerol stocks and competent cells can be found on pages 65–68.

Strain	Deriv.	Genotype	Description/Application	Antibiotic Resistance[1]
AD494	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR ΔmalF3* 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)	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR ΔmalF3* F'[*lac*$^+$(*lacI*^{q})*pro*] *trxB*::kan(DE3)	*trxB*$^-$ expression[3] host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml)
AD494(DE3)pLysS	K-12	*Δara*$^-$ *leu7967 ΔlacX74 ΔphoAPvuII phoR ΔmalF3* F'[*lac*$^+$(*lacI*^{q})*pro*] *trxB*::kan(DE3) pLysS	*trxB*$^-$ high-stringency[3,4] expression host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
B834	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm met*	*met* auxotroph, parent of BL21, control non-expression[2] host	none
B834(DE3)	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm met* (DE3)	met auxotroph parent of BL21, general expression[3] host, ^{35}S-met labeling	none
B834(DE3)pLysS	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm met* (DE3) pLysS	met auxotroph, parent of BL21, high-stringency expression[3,4] host, ^{35}S-met labeling	Chloramphenicol (34 µg/ml)
BL21	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm*	control non-expression[2] host	none
BL21(DE3)	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm* (DE3)	general purpose expression[3] host	none
BL21(DE3)pLysS	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm* (DE3) pLysS (Cm^R)	high-stringency[3,4] expression host	Chloramphenicol (34 µg/ml)
BL21*trxB*(DE3)	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm trxB*15::kan (DE3)	general expression[3] host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml)
BL21*trxB*(DE3)pLysS	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm trxB*15::kan (DE3) pLysS (Cm^R)	high-stringency[3,4] expression host; allows disulfide bond formation in *E. coli* cytoplasm	Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
BLR	B	F$^-$ *ompT hsdS*$_B$ (*r*$_B^-$ *m*$_B^-$) *gal dcm Δ*(*srl–recA*)*306*::Tn*10* (Tc^R)	*recA*$^-$ non-expression[2] host recommended for use with tandem repeats	Tetracycline (12.5 µg/ml)
BLR(DE3)	B	F$^-$ *ompT hsdS*$_B$ (*r*$_B^-$ *m*$_B^-$) *gal dcm Δ*(*srl–recA*)*306*::Tn*10* (Tc^R) (DE3)	*recA*$^-$ expression[3] host recommended for use with tandem repeats	Tetracycline (12.5 µg/ml)
BLR(DE3)pLysS	B	F$^-$ *ompT hsdS*$_B$ (*r*$_B^-$ *m*$_B^-$) *gal dcm Δ*(*srl–recA*)*306*::Tn*10* (DE3) pLysS	*recA*$^-$ high-stringency[3,4] expression host recommended for use with tandem repeats	Chloramphenicol (34 µg/ml) Tetracycline (12.5 µg/ml)
HMS174	K-12	F$^-$ *recA1 hsdR*(*r*$_{K12}^-$ *m*$_{K12}^+$) *Rif*^{R}	control non-expression[2] host	Rifampicin (200 µg/ml)
HMS174(DE3)	K-12	F$^-$ *recA1 hsdR*(*r*$_{K12}^-$ *m*$_{K12}^+$) *Rif*^{R} (DE3)	*recA*$^-$ K-12 expression[3] host	Rifampicin(200 µg/ml)
HMS174(DE3)pLysS	K-12	F$^-$ *recA1 hsdR*(*r*$_{K12}^-$ *m*$_{K12}^+$) *Rif*^{R} (DE3) pLysS	*recA*$^-$ K-12 high-stringency[3,4] expression host	Chloramphenicol (34 µg/ml) Rifampicin (200 µg/ml)
NovaBlue	K-12	*endA1 hsdR17*(*r*$_{K12}^-$ *m*$_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15* ::Tn*10*(Tc^R)]	non-expression[2] host, general purpose cloning, plasmid preps	Tetracycline (12.5 µg/ml)
NovaBlue(DE3)	K-12	*endA1 hsdR17*(*r*$_{K12}^-$ *m*$_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15* ::Tn*10*(Tc^R)] (DE3)	*recA*$^-$ *endA*$^-$ K-12 *lacI*^{q} expression[3] host recommended for use with NovaTope-® System	Tetracycline (12.5 µg/ml)
Origami™ B	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm lacY1 ahpC gor522*::Tn*10* (Tc^R) *trxB*::kan	control non-expression[2] host	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml)
Origami B(DE3)	B	F$^-$ *ompT hsdS*$_B$(*r*$_B^-$ *m*$_B^-$) *gal dcm lacY1 ahpC gor522*::Tn*10* (Tc^R) *trxB*::kan (DE3)	general expression[3] host; contains Tuner *lac* permease mutation and *trxB/gor* mutations for cytoplasmic disulfide bond formation	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml)

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Strain	Deriv.	Genotype	Description/Application	Antibiotic Resistance[1]
Origami B(DE3) pLysS	B	F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm lacY1 ahpC gor522*::Tn*10* (Tc^R) *trxB*::kan (DE3) pLysS (Cm^R)	high-stringency[3,4] expression host; contains Tuner *lac* permease mutation and *trxB/gor* mutations for cytoplasmic disulfide bond formation	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
Origami™ [5]	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$(*lacI^q*)*pro*] *gor522* ::Tn*10* (Tc^R) *trxB*::kan	control non-expression[2] host	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml)
Origami(DE3)[5]	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$(*lacI^q*)*pro*] *gor522* ::Tn*10* (Tc^R) *trxB*::kan (DE3)	general expression[3] host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in *E. coli* cytoplasm	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml)
Origami(DE3)pLysS[5]	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$(*lacI^q*)*pro*] *gor522* ::Tn*10* (Tc^R) *trxB*::kan (DE3) pLysS (Cm^R)	high-stringency[3,4] expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in *E. coli* cytoplasm	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
Rosetta™	B	F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm lacY1* pRARE[6] (Cm^R)	control non-expression[2] host	Chloramphenicol (34 µg/ml)
Rosetta(DE3)	B	F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm lacY1* (DE3) pRARE[6] (Cm^R)	general expression[3] host; *lac* permease mutation allows control of expression level, provides rare codon tRNAs	Chloramphenicol (34 µg/ml)
Rosetta(DE3)pLysS	B	F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm lacY1* (DE3) pLysSRARE[6] (Cm^R)	high-stringency[3,4] expression host; *lac* permease mutation allows control of expression level, provides rare codon tRNAs	Chloramphenicol (34 µg/ml)
RosettaBlue™	K-12	*endA1 hsdR17*($r_{K12}^- m_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA$^+$B$^+$ lacI^{q}ZΔM15* ::Tn*10*(Tc^R)] pRARE[6] (Cm^R)	control non-expression[2] host	Tetracycline (12.5 µg/ml) Chloramphenicol (34 µg/ml)
RosettaBlue(DE3)	K-12	*endA1 hsdR17*($r_{K12}^- m_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA$^+$B$^+$ lacI^{q}ZΔM15* ::Tn*10*(Tc^R)] (DE3) pRARE[6] (Cm^R)	*recA$^-$ endA$^-$* K-12 *lacI^q* general expression[3] host; provides rare codon tRNAs	Tetracycline (12.5 µg/ml) Chloramphenicol (34 µg/ml)
RosettaBlue(DE3) pLysS	K-12	*endA1 hsdR17*($r_{K12}^- m_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA$^+$B$^+$ lacI^{q}ZΔM15* ::Tn*10*(Tc^R)] (DE3) pLysSRARE[6] (Cm^R)	*recA$^-$ endA$^-$* K-12 *lacI^q* high-stringency[3,4] expression host; provides rare codon tRNAs	Tetracycline (12.5 µg/ml) Chloramphenicol (34 µg/ml)
Rosetta-gami™ [5]	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$(*lacI^q*)*pro*] *gor522* ::Tn*10* (Tc^R) *trxB*::kan pRARE[6] (Cm^R)	control non-expression[2] host	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
Rosetta-gami(DE3)[5]	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$(*lacI^q*)*pro*] *gor522* ::Tn*10* (Tc^R) *trxB*::kan (DE3) pRARE[6] (Cm^R)	general expression[3] host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in *E. coli* cytoplasm, provides rare codon tRNAs	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
Rosetta-gami (DE3)pLysS[5]	K-12	*Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac*$^+$(*lacI^q*)*pro*] *gor522* ::Tn*10* (Tc^R) *trxB*::kan (DE3) pLysSRARE[6] (Cm^R)	high-stringency[3,4] expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in *E. coli* cytoplasm, provides rare codon tRNAs	Tetracycline (12.5 µg/ml) Kanamycin (15 µg/ml) Chloramphenicol (34 µg/ml)
Tuner™	B	F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm lacY1*	control non-expression[2] host	none
Tuner(DE3)	B	F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm lacY1* (DE3)	general expression[3] host; *lac* permease mutation allows control of expression level	none
Tuner(DE3)pLysS	B	F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm lacY1* (DE3) pLysS (Cm^R)	high-stringency[3,4] expression host: *lac* permease mutation allows control of expression level	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. pLysE hosts provide even greater stringency; these are available separately as glycerol stocks.
5. The original *trxB/gor* double mutant (Stewart, 1998) required reducing agent in the growth medium to support normal growth rates. The Origami™ strains are a derivative (FA113) of the original strain that carry a mutation (*ahpC*) which allows normal growth rates in the absence of supplemental reducing agent (Bessette et al., 1999; Ritz et al., 2001).
6. pRARE and pLysSRARE encode the tRNA genes *argU*, *araW*, *ileX*, *glyT*, *leuW*, *proL*, *metT*, *thrT*, *tyrU* and *thrU*. The rare codons AGG, AGA, AUA, CUA, CCC, and GGA are supplemented (Novy et al., 2001).

I. 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 table on page 8. Both types of selection have been widely used, but several simple guidelines are recommended when using vectors carrying the β-lactamase gene (see Section V, *Optimizing Expression*). While ampicillin resistance is commonly used for selection in a variety of cloning vectors, kanamycin resistance may be preferable under certain conditions, such as for protein expression in laboratories requiring GMP standards and when subcloning target genes from other ampicillin-resistant vectors. Ampicillin selection tends to be lost in cultures because secreted β-lactamase and the drop in pH that accompanies bacterial fermentation both degrade the drug. 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 most of the amp^R pET vectors involves the direction of transcription of the drug resistance gene. In kan^R pET vectors, the *kan* gene is in the opposite orientation from the T7 promoter, so induction of the T7 promoter should not result in an increase in *kan* gene product. In contrast, in most amp^R pET vectors the β-lactamase gene is located downstream and in the same orientation as the T7 promoter. All pET translation vectors have the native T7 transcription terminator (Tϕ) located before the β-lactamase gene. However, this terminator is only approximately 70% effective, allowing T7 RNA polymerase read-through to produce a small amount of β-lactamase RNA in addition to the target RNA. This results in the accumulation of β-lactamase enzyme in induced cultures. Accordingly, the orientation of the β-lactamase gene has been reversed in the pET-43.1 and pET-44 vectors, so that read-through by the T7 RNA polymerase will not result in increased levels of β-lactamase gene product.

J. Bacteriophage CE6

Another alternative for expression of 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 an appropriate host, the newly made T7 RNA polymerase transcribes target DNA so actively that normal phage development cannot proceed. Although this method is less convenient than induction of DE3 lysogens, it can be used if target gene products are too toxic to be maintained any other way. No T7 RNA polymerase is present in the cell before infection, so it should be possible to express any target DNA that can be cloned under control of a T7 promoter in this way. Bacteriophage CE6 is available separately from Novagen (see Technical Bulletin 007).

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K. 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 induction controls are not suitable for cloning. 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, N, and O.1, which contain no insert). The following table lists the various induction control strains and matching pET vectors. See *Induction Control* on page 57 for more details on the β-galactosidase assay.

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

Abbreviations: amp = ampicillin or carbenicillin, kan = kanamycin, cam = chloramphenicol, tet = tetracycline

T = thrombin, X = Factor Xa, E = enterokinase

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

II. Getting Started

A. Overview of The pET System Process

Process	Detail
Prepare pET Vector	1. Digest with restriction enzyme(s) and dephosphorylate, or use LIC vector 2. Gel purify (or use LIC vector)
↓ **Prepare Insert DNA**	1. Plasmid prep and/or PCR 2. Restriction digest or generate LIC overhang 3. Gel purify
↓ **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, prepare plasmid DNA, verify reading frame by sequencing, or *in vitro* transcription/translation
↓ **Transform into Expression Host**	1. Transform host carrying T7 RNA polymerase gene (λDE3 lysogen) or non-DE3 host compatible with λCE6 infection
↓ **Induce and Optimize Expression of Target Protein**	1. Optional: test the 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
↓ **Scale-up** ↓ **Purify Target Protein**	1. Scale up culture 2. Prepare extract 3. Affinity purify 4. Cleave tags and remove protease (if desired)

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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 include LB, TB ('terrific broth"), M9 and M9ZB. Recipes and stock solutions are shown below and on the following page.

LB	
Per liter: 10 g Bacto® tryptone 5 g Yeast extract 10 g NaCl • Adjust pH to 7.5 with 1N NaOH • Autoclave	
TB (Sambrook et al., 1989)	**K phosphate**
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	Per liter: 23.1 g KH_2PO_4 125.4 g KH_2PO_4 • Autoclave
M9	**20X M9 salts**
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_20	Per liter: 20 g NH_4Cl 60 g KH_2PO_4 120 g $Na_2HPO_4•7H_20$ • Autoclave
M9ZB (Studier et al., 1990)	**10X M9 salts**
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)	Per liter: 10 g NH_4Cl 30 g KH_2PO_4 60 g $Na_2HPO_4•7H_20$ • Autoclave

Stock solution	Preparation	Cat. No.
100 mM IPTG (isopropyl β-D-thiogalactopyranoside)	2.38 g IPTG in 100 ml deionized water. Filter sterilize and store at –20°C.	70527-3*
Carbenicillin (disodium salt)	50 mg/ml in deionized water. Store at –20°C. Use at 50 µg/ml.	69101-3
Ampicillin (sodium salt)	25 mg/ml in deionized water. Store at –20°C. Use at 50 µg/ml.	171254 (Calbiochem)
Chloramphenicol	34 mg/ml in ethanol. Store at –20°C. Use at 34 µg/ml.	220551 (Calbiochem)
Kanamycin (sulfate)	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*, Origami™, Origami B, Rosetta-gami™).	420311 (Calbiochem)
Tetracycline	5 mg/ml in ethanol. Store at –20°C. Use at 12.5 µg/ml.	58346 (Calbiochem)
Rifampicin	10 mg/ml in 67% methanol, 0.17 N NaOH. Use at 200 µg/ml within 5 days. Protect from light.	557303 (Calbiochem)
Glucose	20% (w/vol) D-glucose solution in H_20. Autoclave. Store sterile solution at room temperature. Add glucose to LB agar with antibiotics to a final concentration of 0.5–1% (see page 12).	346352 (Callbiochem)

*100 mM IPTG Solution

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 ml 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*, page 34).

To inoculate a culture from the frozen stock:

1. Scrape or melt a few microliters from the surface (use a sterile pipet tip or plastic culture loop).
2. Streak on an agar plate or inoculate liquid medium [containing appropriate antibiotic(s)].
3. Return the remainder to the –70°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.

- 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 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 (Calbiochem Cat. No. 524576) 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. Novagen's SpinPrep™ Gel DNA Kit is ideal for rapid isolation of DNA fragments from agarose gel slices.

To digest and gel-purify the vector:

1. Assemble the following components in a microcentrifuge tube:

3 µg	pET vector
3 µl	10X restriction enzyme buffer
10–20 U	Each restriction enzyme (assuming compatible 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 (Calbiochem Cat. No. 524576) 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 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. 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. Avoid over exposure to the light source, which can cause nicks and double strand breaks in the DNA.
8. Recover the DNA from the gel slice. The SpinPrep™ Gel DNA Kit (Cat. No. 70852-3) is ideal for this application. Resuspend the final product in a total volume of 30 µl (usually about 50 ng/µl DNA). The DNA can be quantified spectophotometrically or using the PicoGreen kit from Molecular Probes. 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 TE-buffered phenol:CIAA (1:1; CIAA is chloroform:isoamyl alcohol, 24:1), and 1 volume CIAA. Then precipitate with 0.1 volume 3 M Na acetate and 2 volumes of ethanol. Centrifuge at 12,000 x g for 10 min, rinse the pellet with 70% ethanol, air dry, and resuspend in 30 µl TE buffer. Pellet Paint® Co-Precipitant (2 µl) could be added to the precipitation for easy visualization of the pellet.

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 or LIC overhangs, and (3) place the coding region in the proper reading frame. In general, primers should contain a minimum of 15 (preferably 18–21) nucleotides complementary to the sequence of interest with a GC content of 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 high fidelity, such as KOD HiFi, Hot Start, or XL DNA Polymerases.
- Limit the number of PCR cycles.
- Increase the concentration of target DNA.
- Increase the primer concentration.

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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. 70526-3) 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 (see Technical Bulletin 233). 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.

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) or use the Clonables™ 2X Ligation Premix (Cat. No. 70573-3). 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 (an expression strain) 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.

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 preparation 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. The standard transformation reaction requires 20 µl cells, so each tube contains enough cells for 10 transformations. Singles™ Competent Cells are provided in 50 µl aliquots, which are used "as is" for single 50 µl

transformations. Note that there are a few steps in the protocol that vary for the Singles™ vs. standard cells. Novagen's NovaBlue and BL21(DE3) Competent Cells are also offered in a high-throughput 96-well plate format known as HT96™ Competent Cells (see Technical Bulletin 313).

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™ Competent 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

Prepare LB agar plates with appropriate antibiotic ahead of time

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is surrounded by ice. If the standard cells are to be used, 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.
3. <u>*Standard Competent Cells*</u>:
   Pipet 20 µl aliquots of cells into the pre-chilled tubes.

   <u>*Singles Competent Cells:*</u>
   Proceed to Step 4 or 5, depending on whether a Test Plasmid sample is included as a positive control.
4. (Optional) To determine transformation efficiency, add 0.2 ng (1 µl) Test Plasmid provided with Competent Cells to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
5. Add 1 µl of a ligation reaction or purified plasmid DNA directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is surrounded by ice except for the cap. Repeat for additional samples.
6. Incubate the tubes on ice for 5 min.
7. Place the tubes in a 42°C water bath for exactly 30 sec; do not shake.
8. Place the tubes on ice for 2 min.
9. <u>*Standard Competent Cells:*</u>
   Add 80 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.

   <u>*Singles Competent Cells:*</u>
   Add 250 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
10. Selection for transformation is accomplished by plating on medium containing antibiotic for the plasmid encoded drug resistance. Additional host-specific antibiotics may also be appropriate to ensure maintenance of the host feature(s) (see chart on page 15).
    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. If selecting for kanamycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating.

    When using strains other than NovaBlue: shake at 37°C (250 rpm) for 60 min prior to plating.

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.

11. Spread 5–50 µl of each transformation on LB agar plates containing the appropriate antibiotic for the plasmid and host strain (see pages 8 and 16). 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 × 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 (e.g., 5 µl of NovaBlue cells at 1 × 10^8 efficiency) or 10 µl (e.g., 10 µl of cells at 1 × 10^6 efficiency) of the final transformation mix in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the amp^R gene).*

12. 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 techniques

1. Remove the plates from the incubator. If plating less than 25 µl of the transformation, we recommend plating onto a pool of SOC, which facilitates even colony distribution on the plate surface. Using a sterile pipet tip, place 40–60 µl of SOC in the center of a plate for a plating cushion.
2. To remove the transformation sample, 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.)

ColiRollers™ Plating Beads are treated glass beads that eliminate the use of the spreader and alcohol flame while evenly and consistently distributing cells without damage.

ColiRollers™ Plating Beads

To use ColiRollers, simply dispense 10–20 beads per plate. The beads can be dispensed before or after pipetting the transformation mix on the plate. Cover the plate with its lid and move the plate back and forth several times. The rolling action of the beads distributes the cells. Several plates can be stacked up and shaken at one time. After all plates have been spread, discard the ColiRollers by inverting the plate over a collection container. Cover and incubate (step 12 above).

Standard spreader

Completely immerse the plating spreader (bent glass rod or equivalent) into ethanol and flame to sterilize. After the flame is extinguished, allow the spreader to cool ~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.

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.*

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. After 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, because 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.

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 preparation 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 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. Consult the appropriate vector map for all available primers. Alternatively, just the vector-specific primers can be used in one reaction if insert orientation information is not desired.

Important: To achieve efficient amplification of entire CBD•Tag™ sequences in pET CBD vectors with NovaTaq™ DNA Polymerase, 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.

Transcription/translation analysis with the EcoPro™ System

pET constructs can be quickly evaluated for expression of the desired target protein by using the EcoPro T7 Coupled Transcription/Translation System. The EcoPro System employs a proprietary fractionated *E. coli* extract to produce greater yield of full-length proteins as compared to other prokaryotic *in vitro* expression systems. EcoPro is designed for efficient *in vitro* synthesis of proteins directly from supercoiled or linear DNA templates, including PCR products (McCormick and Ambuel, 2000). PCR products can be prepared using the ligation reaction or colonies as the template. 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. The synthesized proteins may be detected by labeling with ^{35}S-methionine, by Western blotting using tag-specific or target protein specific antibodies, or quantitative assay using FRETWorks™ S•Tag™ or S•Tag Rapid Assay Kits.

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Plasmid templates

DNA templates intended for use with the EcoPro™ T7 System must be substantially free of contaminating RNase, Mg^{2+} and salts. Plasmids isolated using the Mobius™ or UltraMobius™ Plasmid Kits are essentially RNase-free and can be used directly in the transcription/translation reactions without further purification. Plasmid DNA prepared with other protocols using RNase A should be phenol extracted and precipitated. Add TE to 100 µl and extract twice with TE-buffered phenol:CIAA (1:1; CIAA is 24 parts chloroform, 1 part isoamyl alcohol) and once with CIAA. Transfer the final aqueous phase to a fresh tube and add 0.1 volume 3 M Na acetate and 2 volumes ethanol. Mix and 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® or Pellet Paint NF Co-Precipitant can be added along with the TE buffer prior to extraction to facilitate recovery of the DNA (the –20°C incubation can be eliminated if using Pellet Paint).

Note: *Unlike Novagen's Mobius and UltraMobius Plasmid Kits, some commercial plasmid DNA isolation kits do not remove all the RNase used during purification; therefore, when using other kits it is usually safest to extract the DNA with phenol as described above.*

If linearized plasmid is to be used as template, we recommend using restriction enzymes that leave either blunt ends or 5' overhangs. Templates containing 3' overhangs can cause aberrant transcription from the non-coding strand by T7 RNA polymerase (Schenborn and Mierendorf, 1985). The antisense RNA will anneal with the desired transcripts and potentially inhibit translation in the EcoPro reaction. If an enzyme that produces 3' overhangs cannot be avoided, the DNA can be treated with the Klenow fragment of DNA polymerase in the presence of 25 µM dNTPs (5 min, 25ºC, 1 U/µg DNA) to blunt the ends prior to use. Commonly used restriction enzymes that produce 3' overhangs include *Pst* I, *Kpn* I, *Sac* I, *Sac* II, *Bst*X I, *Nsi* I, *Apa* I, and *Aat* II.

PCR templates

To prepare suitable PCR products for transcription/translation, use primers that allow amplification of the T7 promoter along with the correct orientation of the target insert (see table below). 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 is designed for this purpose and is suitable for most pET vectors. 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). However, the T7 Terminator Primer is suitable if directionality is not required.

Primers for *in vitro* transcription/translation analysis

Vector	5' primer	3' primer[2]
pET series[1]	pET Upstream Primer	T7 Terminator Primer
pET LIC Vectors	pET Upstream Primer	T7 Terminator Primer

[1] except for pET-17b, 20b(+), 23(+) and 23a-d(+); use plasmid DNA as a template

[2] Vector-specific 3' primers will amplify both insert orientations, if present

Important: *Glycerol must be added to the PCR reaction mixture to a final concentration of 8–10% for amplification of CBD•Tag™ constructs with the pET upstream primer using NovaTaq™ DNA Polymerase.*

Ligation PCR for transcription/translation analysis

A ligation reaction can be analyzed directly by PCR followed by EcoPro™ T7 transcription/translation. Note that this approach will not verify individual clones; for this purpose colony PCR should be used.

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 Nova*Taq*™ Buffer with $MgCl_2$
1 µl	pET Upstream Primer (5 pmol)
1 µl	downstream primer (5 pmol)
1 µl	10 mM dNTP Mix
0.25 µl	(1.25 U) Nova*Taq* DNA Polymerase
x µl	Sterile deionized water to volume
50 µl	Total volume

Note: For greatest specificity, and yield of long complex targets use KOD Hot Start and KOD XL DNA Polymerases, respectively, with their buffers and cycling conditions (see Technical Bulletin 341, 342). KOD polymerases are not available for sale in Japan through Novagen.

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* on page 32 for details). A strong band should appear that has a size corresponding to the distance between (and including) the primers.
5. Prior to use in EcoPro™ T7 reactions, PCR products should be precipitated to remove salts. To precipitate a 50 µl PCR reaction, add 5.2 µl 3 M sodium acetate and 115 µl 95% ethanol. To visualize the pellet, add 2 µl of Pellet Paint® (Cat. No. 69049-3) or Pellet Paint NF (70748-3) Co-Precipitant which is compatible with the EcoPro T7 reaction. Vortex briefly and spin at 14,000 × g for 5 min. Wash the pellet briefly with 70% ethanol, followed by 100% ethanol. Dry the pellet to remove residual ethanol and resuspend in 10–20 µl deionized water. Use 2–4 µl in the EcoPro T7 reaction.

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Colony PCR for transcription/translation 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.
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. Assemble the following components for colony PCR.

   Per reaction:

31.75 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 Nova*Taq*™ Buffer with $MgCl_2$,
0.25 µl	(1.25 U) Nova*Taq* DNA Polymerase
40 µl	Total volume

   To account for pipetting losses, it is convenient to multiply the amounts by X.5, where X is the number of reactions.

Note: For greatest specificity, and yield of long complex targets during PCR use KOD Hot Start and KOD XL DNA Polymerases respectively, with their buffers and cycling conditions (see Technical Bulletin 341, 342). KOD polymerases are not available for sale in Japan through Novagen.

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* on page 32 for details).
10. Prior to use in EcoPro™ T7 reactions, PCR products should be precipitated to remove salts. To precipitate a 50 µl PCR reaction, add 5.2 µl 3 M sodium acetate and 115 µl 95% ethanol. To visualize the pellet, add 2 µl of Pellet Paint® (Cat. No. 69049-3) or Pellet Paint NF (Cat. No 70748-3) Co-Precipitant which is compatible with the EcoPro T7 reaction. Vortex briefly and spin at 14,000 × g for 5 min. Wash the pellet briefly with 70% ethanol, followed by 100% ethanol. Dry the pellet to remove residual ethanol and resuspend in 10–20 µl deionized water. Use 2–4 µl in the EcoPro T7 reaction.

Colony screening

Colonies can be screened for inserts without plasmid preparation 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. Exceptions are pET-17xb, pSCREEN-1b(+) and pEX*lox*®(+) vectors, in which the T7 gene *10* primer is recommended over the T7 promoter primer and pET-43.1a-c(+) and pET-44a-c(+), in which the Nus•Tag™ primer is recommended over the T7 promoter primer.

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

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.

Plasmid preparation procedure

After positive clones are identifed, mid-copy pET plasmid DNA can be isolated for transformation into expression hosts, restriction mapping, and sequence analysis. Plasmid DNA from candidate recombinants may also be evaluated using *in vitro* transcription/translation analysis. It is important that the template be RNase-free for *in vitro* transcription and translation. Plasmid DNA isolated with Mobius™ or UltraMobius™ Kits are essentially RNase-free. However, plasmid DNA isolated with SpinPrep™ Plasmid Kits or other manufacturers kits may require an additional phenol:CIAA extraction to eliminate RNases as described on page 28.

Mid copy refers to a pBR322 origin yielding 20–60 copies per cell. High-copy refers to a pUC origin yielding > 200 copies per cell.

Plasmid Preparation Kit	Scale	DNA Yield	Cat. No.	Size
Mobius 1000 Plasmid Kit	100 ml (high-copy) 250 ml–1.5 liter (mid-copy)	> 1 mg (high-copy) 200 µg–1 mg (mid copy)	70854-3 70853-3 70853-4	2 rxn* 10 rxn* 25 rxn*
UltraMobius 1000 Plasmid Kit	100 ml (high-copy) 250 ml–1.5 liter (mid-copy)	> 1 mg (high-copy) 200 µg –1 mg (mid copy)	70907-3 70906-3 70906-4	2 rxn* 10 rxn* 25 rxn*
Mobius 500 pET Plasmid Kit	500 ml culture	500 µg (low-copy)	70969-3	10 rxn
Mobius 200 Plasmid Kit	35 ml culture (high-copy or mid-copy)	> 200 µg (high-copy) > 30 µg (mid-copy)	70970-3	25 rxn
UltraMobius 200 Plasmid Kit	35 ml culture (high-copy or mid-copy)	> 200 µg (high-copy) > 30 µg (mid-copy)	71090-3	25 rxn
SpinPrep Plasmid Kit	1–3 ml culture	5–10 µg (high-copy) 0.25–1 µg (mid-copy)	70957-3 70851-3	20 rxn 100 rxn

*The kit sizes described are for the 100 ml (high-copy) or 250 ml (mid-copy) preparations. Additional buffers are required for > 250 ml (mid-copy) scale (Technical Bulletin 279).

If the sequencing template is precipitated, the addition of Pellet Paint® or Pellet Paint NF Co-Precipitant helps make the pellet visible after precipitation. Use Pellet Paint NF (Cat. No. 70748-3) with rhodamine based labeling methods (e.g. PE Applied Biosystems automated sequencers) and Pellet Paint (Cat. No. 69049-3) with Cy5-based automated sequencers.

Sequencing

Detailed protocols for sequencing with double stranded and single stranded templates are available from many manufacturers of sequencing kits. Primers for sequencing are indicated on the pET vector maps available at www.novagen.com.

It is possible to prepare single stranded DNA template from PCR products with the Strandase™ Kit (Cat. No. 69202-3) or from pET plasmids that carry the phage f1 origin of replication by infection with single stranded helper phage. The f1 origin in pET vectors is oriented such that the single stranded DNA produced will anneal with the T7 terminator primer. The required helper phage (strain R408 or 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.

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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 page 25. Streak transformants for single colonies and prepare glycerol stocks as described on page 22.

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 for full induction, while 1 mM IPTG is recommended for full induction 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, *Target Protein Verification* (page 52).

Some λDE3 host strains allow variation of the expression level simply by varying the concentration of IPTG added to induce expression. The Rosetta™(DE3), Tuner™(DE3) and Origami™ B(DE3) strains contain the *lacY1* mutation eliminating the active transport of lactose into cells via *lac* permease. Therefore, these strains are less sensitive to lactose in the media and IPTG induction results in a more uniform entry into all cells in the population. When using these strains, a range of IPTG concentrations from 25 µM to 1 mM should be tested, and the induced cultures examined for activity and solubility of the target protein to establish the optimal IPTG concentration for the desired result.

Preparation for induction

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

Alternatively, inoculate a single colony or a few microliters from a glycerol stock into 2 ml LB medium containing the appropriate antibiotic for the plasmid and host strain. 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.

Overnight cultures should be avoided to prevent elevated levels of basal expression (see page 22) and depletion of the antibiotic from the media (see page 38). However, if cultures are grown overnight, 0.5–1% glucose may be added to the media in order to reduce target protein expression prior to induction.

Sample induction protocol

1. Incubate with shaking at 37°C until OD_{600} reaches 0.4–1 (0.6 recommended; about 3 h).
2. (Optional) Remove samples for the uninduced control and for titering (not recommended with the T7*lac* promoter plasmids) as described in *Plasmid Stability Test* (page 38). 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, *Target Protein Verification*, 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.

V. Optimizing Expression

The following sections describe 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.

A. Enhancing Solubility and Folding

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

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

Temperature

Growth at 37°C causes some proteins to accumulate as inclusion bodies, while incubation at 30°C 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.

Lysis buffer

The partitioning of a given target protein into the soluble or insoluble fraction can be strongly influenced by the nature of the lysis buffer. Proteins containing hydrophobic or membrane-associated domains may partition into the insoluble fraction when using a standard lysis buffer, such as 1X His•Bind® Binding Buffer (which contains 500 mM NaCl), but may not actually be present in inclusion bodies. Proteins in the insoluble fraction due to association with bacterial lipids or membranes may often be converted to the soluble fraction by adding millimolar amounts of nonionic or zwitterionic detergents to the lysis buffer. BugBuster® Protein Extraction Reagent or PopCulture™ Reagent, both used in combination with rLysozyme™ Solution, can be an effective choice to consider for solubilization. The proprietary formulations utilize a nonionic and zwitterionic detergent cocktail capable of solubilizing cell wall and membrane components, thereby releasing cellular proteins without denaturation. The detergents in BugBuster and PopCulture may also facilitate solubilization of other membrane bound or hydrophobic proteins. Other detergents may be needed to solubilize these membrane proteins and it is possible many may not be solubilized. Choosing a detergent for solubilization remains an empirical task. For a review of the use of detergents in bacterial lysis, see “Experiment 2: Solubilization and Purification of the Rat Liver Insulin Receptor” (Brennan 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 or digesting the nucleic acid with a nuclease such as Benzonase® Nuclease (see Technical Bulletin 261).

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Periplasmic localization

An alternative strategy to obtain active, soluble proteins is to use vectors that enable export into the periplasm, which is a more favorable environment for folding and disulfide bond formation (Rietsch et al., 1996; Raina and Missiakas, 1997; Sone et al., 1997). For this purpose vectors carrying signal peptides are used, such as pET-12, 20, 22, 25, 26, 27, 36, 37, 38, 39 and 40. Target proteins exported to the periplasm with the CBD_{cenA} signal sequence may leak to the medium for simplified purification (Novy et al., 1997). However, some target proteins will not be good candidates for periplasmic localization. For example, some fusions of β-gal to a periplasmic protein have proven to be toxic (Snyder and Silhavy, 1995). In addition, the net charge of the N-terminal amino acids on the mature protein can inhibit translocation (Kajava et al., 2000)

While several pET vectors contain signal sequences for fusion with target genes, pET-39b(+) and pET-40b(+) are designed to create fusions to the enzymes that catalyze the formation (DsbA) or isomerization (DsbC) of disulfide bonds in the periplasm (Missiakas et al., 1994; Zapun et al., 1995). If a fusion protein is competent to localize to the periplasm, then its direct association with the catalytic enzyme may enhance its solubility and facilitate disulfide bond formation. A properly folded fusion protein requiring formation of disulfide bonds for activity has been isolated following fusion to DsbA (Collins-Racie et al., 1995). Note that 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® or Ni-NTA His•Bind chromatography. Prior to exposing the fusion protein to a reducing agent, either EDTA should be added to a final concentration of 1 mM, or the sample should be dialyzed to remove residual Ni^{2+}. EDTA and dialysis is probably unnecessary if Ni-NTA His•Bind resin was used for purification.

Cytoplasmic localization

The Trx•Tag™, GST•Tag™, and Nus•Tag™ fusion tags are highly soluble polypeptides that can potentially enhance solubility of target proteins. When using vectors designed for cytoplasmic expression, folding can be improved in hosts that are permissive for the formation of disulfide bonds in the *E. coli* cytoplasm (see *Host Strains*, below). Recognition sequences for site specific proteases are engineered into all these vectors for complete removal of the tags (see page 10).

Schistosomal glutathione-S-transferase (GST) is commonly used as an N-terminal fusion partner when expressing proteins in *E. coli*. Although not specifically designed for this purpose, the GST•Tag sequence has been reported to enhance the solubility of its fusion partners. The pET-41a-c(+) and -42a-c(+) vectors encode the GST•Tag sequence that can be cleaved with thrombin or enterokinase and Factor Xa respectively. Note that these vectors carry kanamycin resistance so are not recommended for use with *trxB* mutant hosts.

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

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

Host strains

Many proteins require disulfide bonds for proper folding and activity; however, the cytoplasm of *E. coli* is not a favorable environment for disulfide bond formation. The use of Origami™, Origami B, Rosetta-gami™, AD494, or BL21*trxB* host strains promote the formation of disulfide bonds in the *E. coli* cytoplasm, which may affect the solubility and/or activity of a given target protein. The thioredoxin reductase (*trxB*) mutation is found in the AD494 and BL21*trxB* strains. The Origami, Origami B and Rosetta-gami strains carry the *trxB* mutation plus the glutathione reductase (*gor*) mutation, further increasing disulfide bond formation. If the target protein contains disulfide bonds and the target gene encodes rare codons, the Rosetta-gami strain may be optimal (see below, *Correcting for rare codon usage*). If your target protein contains one or more essential disulfide bonds, the combination of a pET-32a-c(+) vector and a *trxB* or *trxB/gor* host may prove to be optimal because disulfide bond formation in the cytoplasm appears to be dependent on the presence of thioredoxins (Stewart, 1998). Also review *Disulfide bond formation and solubility enhancement* on page 15.

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

B. Correcting for Rare Codon Usage

Most amino acids are encoded by more than one codon, and analysis of *E. coli* codon usage reveals that several codons are underrepresented. In particular, Arg codons AGA, AGG, CGG CGA, Ile codon AUA, Leu codon CUA, Gly codon GGA and Pro codon CCC are rarely used. The tRNA population closely reflects the codon bias of the mRNA population and when the mRNA of heterologous target genes is overexpressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. Insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshifting and amino acid misincorporation.

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

The Rosetta™ strains are designed to enhance expression of target proteins that contain rare Arg codons AGG and AGA, Ile codon AUA, Leu codon CUA, Pro codon CCC, and Gly codon GGA. The tRNA's are expressed from their native promoters on a chloramphenicol resistant plasmid (pACYC backbone) compatible with pET vectors. The Rosetta strains containing pLysS have the rare codon tRNA's on the same backbone as the T7 lysozyme. The Rosetta strain is a B-strain derivative with *lon* and *ompT* protease deficiencies. RosettaBlue™ and Rosetta-gami™ strains are derived from and contain the features of the corresponding K-12 NovaBlue and Origami strains, respectively. RosettaBlue has the added benefits of high transformation efficiency and stringency due to the high level of *lac* repressor ($lacI^q$). The Rosetta-gami strain is a *trxB/gor* mutant enhancing protein folding through the formation of disulfide bonds (see *Disulfide bond formation and solubility enhancement*, page 15). The Rosetta strains are well-

suited to enhance protein expression from target genes containing rare *E. coli* codons that would other wise impede translation (Novy et al., 2001).

C. Toxic Genes and Plasmid Instability

Plasmid pBR322 and many of its derivatives (including pET vectors) are relatively stable and are retained by a very high fraction of host cells even after growth for many generations in the absence of a selective antibiotic. However, problems of plasmid instability can arise when a gene whose product is toxic to the host cell is cloned in the plasmid. The pETcoco™ System reduces background expression to the lowest levels by reducing the copy number of the pETcoco plasmid to one copy per cell (Sektas and Szybalski, 2002). The most toxic gene products may be stabilized and expressed in this system (see Technical Bulletin 333).

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

Use of ampicillin

Use of ampicillin as a selective antibiotic requires special care because 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 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.

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. Also, avoid growing the amp^R cultures into saturation phase (overnight; 16 h) to maintain maximum selection. A more detailed discussion of the potential advantages of kan^R compared to amp^R is presented in *Antibiotic Resistance*, page 18.

Supplementing with glucose

Plasmids containing toxic genes may be destabilized in λDE3 lysogens during overnight cultures due to cAMP stimulation of T7 RNA polymerase (Grossman et al., 1998). This can be effectively eliminated by avoiding overnight cultures or delayed by including 0.5–1% glucose in the culture medium. Review *Media containing glucose*, page 12.

Plasmid stability test

The plasmid stability test determines what proportion of the cells maintain the target plasmid and the ability to express the target protein. This test is only appropriate for pET plasmids with a "plain" T7 promoter and host strain lacking pLysS or pLysE. This test is not reliable with the *T7lac* promoter and the pLysS and pLysE plasmids since colonies that retain the plasmid and successfully express the (innocuous) target protein can still grow in the presence of IPTG. IPTG does not prevent colony formation 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.

A combination of lack of selection (especially with amp^R plasmids) and toxicity of the target protein can lead to an accumulation of cells in the culture that no longer maintain or express the target protein. 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. 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.

Immediately before induction, we recommend testing the culture to determine the fraction of cells that carry the target plasmid on four plates:

Plate #	Instructions	Interpretation
1	plate at a 10^{-6} dilution on agar	All viable cells grow.
2	plate at a 10^{-6} dilution on antibiotic	Only cells that retain the plasmid grow.
3	plate at a 10^{-5} dilution on 1 mM IPTG	Only cells that cells that have lost the plasmid or mutants that have lost the ability to express target DNA will grow.*
4	plate at a 10^{-5} dilution on 1 mM IPTG and antibiotic	Only mutants that retain the plasmid but have lost the ability to express the target DNA will grow.*

* Interpretation of plates 3 and 4 is not reliable when using T7*lac* promoters and pLysS and pLysE plasmids or with pET-3a-c.

In a typical culture useful for producing target proteins, almost all cells will form colonies on the 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. The antibiotic should prevent colony formation by any cell that has lost the target plasmid. The IPTG should prevent colony formation by any cell that has both the inducible gene for T7 RNA polymerase and a functional target plasmid. 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 little or no target protein will be expressed. In this case, cultures should be grown from a dilution of 10^4 or higher from the freezer stock in 8% glycerol as described in the following section and grown directly to the density used for expression.

Stabilize a toxic gene in an amp^R pET vector for glycerol stock storage

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

Storage of ampicillin-resistant strains:

1. Inoculate a colony from the transformation plate into 2 ml LB + 50 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.

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

3. As soon as the colony develops (usually overnight at 37°C), inoculate into 2 ml LB + 50 µg/ml carbenicillin and grow until OD_{600} = 0.5.
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 (page 34) at the time of freezing to determine what fraction of the cells contain functional target plasmid.

Stabilize a toxic gene in amp^R pET vector during induction

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.

D. Other Factors Influencing Expression

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

N-end rule

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

The relationship between a protein's amino terminal amino acid and its stability in bacteria is determined by the N-end rule (Tobias et al., 1991). They reported protein half-lives of only 2 minutes when the following amino acids were present at the amino terminus: Arg, Lys, Phe, Leu, Trp, 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 targeted for rapid degradation. Therefore, when a *Nde* I site is employed for the production of unfused target proteins from pET vectors, Leu codons in the penultimate position should be avoided. Leu codons in this position are not available when using *Nco* I as the cloning site, because the penultimate codon must begin with G.

Secondary site translation initiation

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

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Secondary structure in the mRNA transcript

Secondary structure in the mRNA transcript can interfere with the AUG translation initiation codon and/or the ribosome binding site (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.

Unexpected stop codons

Unexpected stop codons can be generated by mutation, especially when cloning PCR products. Sequencing can reveal these mutations, but another alternative is to test the construct's ability to produce the target protein by *in vitro* translation. A very convenient test is done using Novagen's EcoPro™ T7 System (see page 28).

Transcription terminator

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 many amp^R pET vectors. Because Tϕ reduces the amount of this competing mRNA, it allows more target protein to be made. In all the kanamycin-resistant pET vectors and ampicillin-resistant pET-43.1 and pET-44 series, the kan or amp genes and the target gene have opposite orientations so no competing mRNAs are known to be made along with the target mRNA.

Instability of the target mRNA and protein

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

VI. Target Protein Verification

This section describes target protein isolation methods for analysis and purification. With each target protein, it is important to verify the production, localization and estimate the yield in the culture medium or cell. To facilitate verification, a small-scale analysis of total cell protein and the four fractions in the following sequence medium, periplasm, soluble cytoplasm, insoluble cytoplasm is recommended and allows the material from one fraction to be used to prepare the next. Results of this analysis may lead to further optimization of the induction conditions or large-scale induction and purification using one or several of the protein extraction techniques described in this section.

In addition to analyzing the four fractions, a quick screen analysis of induced cultures with PopCulture® Reagent may be desirable especially when screening numerous clones. The PopCulture quick screen allows for rapid characterization of target protein activity and analysis of expression levels directly in the culture medium without cell harvest.

Normalizing loading volumes for SDS-PAGE

To facilitate the gel and Western analysis, two worksheets are provided to record data and calculate normalized loading volumes for standard mini gels (page 51). This formula relies on the generation of accurate harvest OD_{600} readings and the determination of concentration factors for the fractions generated. The sample concentration factor represents the volume of original culture used to produce the fraction divided by the final volume of the fraction.

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. Add the entire 3 ml culture to 100 ml medium containing antibiotics. This scale is convenient for initial analysis of the four fractions but may be adjusted as desired.
3. Shake the 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. Add IPTG to one of the 50 ml cultures and the other culture will serve as an uninduced control. For plasmids having the T7*lac* promoter, add IPTG to 1 mM (500 µl of sterile 100 mM IPTG) or for "plain" T7 promoter vectors, use 200 µl IPTG for a final concentration of 0.4 mM. Or vary the IPTG concentrations with *lacY* mutant strains (Tuner, Rosetta-gami, and Origami B). Incubate both cultures with shaking at the desired temperature for the appropriate amount of time. Note that when directing fusion proteins to the periplasmic space, leakage of the protein to the medium might be enhanced by prolonged inductions (16 h or 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 (page 51).

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

Prior to harvesting the cells, target protein activity and expression levels can be quickly assessed directly without centrifuging the cells using PopCulture Reagent. In addition, the prepared extract can be centrifuged to analyze separate soluble and insoluble fractions.

1. After induction, remove a 1 ml sample and add 0.1 volume (100 µl) of PopCulture Reagent.
2. Add 40 U (1 µl of a 1:750 dilution) rLysozyme™ Solution per 1 ml of original culture volume to enhance cell lysis. Addition of rLysozyme is not necessary if the host strain contains pLysS or pLysE.
   Optional:
   Add 1 µl (25 U) Benzonase® Nuclease per 1 ml of original culture volume to degrade DNA and RNA for a non-viscous sample. rLysozyme and Benzonase can be pre-mixed with PopCulture prior to use.
3. Incubate 10–15 min at room temperature.
4. Assay for expression levels, target protein activity and solubility.
   SDS-PAGE of total cell protein: Combine an aliquot of the prepared extract with an equal volume 4X SDS Sample Buffer (Cat. No. 70607-3) for detection with Coomassie staining or Western blotting. Given an OD between 3–5, a highly expressed protein may be detected with Coomassie staining using a 10 µl sample. Low expression levels and cell densities may require Western blotting.
   Quantitative assay: Fusion tag specific quantitative assays such as FRETWorks™ S•Tag™ Assay, S•Tag Rapid Assay, and GST•Tag™ Assay are compatible with the PopCulture extracts, as are Bradford and BCA protein assays.
   Target protein specific assays: Because proteins generally retain their activities and conformation, protein specific activity and immunoassays are likely to be compatible with PopCulture extraction.
   Solubility: To assess soluble and insoluble fractions, centrifuge the crude extract at 14,000 × g for 10 min to separate the fractions. *Soluble fraction*: An aliquot of the supernatant representing the soluble fraction can be assessed on by SDS-PAGE (as described above) or protein activity and quantitative assays may be performed. *Insoluble fraction:* Resuspend the pellet in 1 ml 1% SDS with heating and vigorous mixing or sonication. An aliquot of the solubilized pellet representing the insoluble fraction can be assessed by SDS-PAGE (as described above) or assayed using the S•Tag Rapid Assay or FRETWorks S•Tag Assay (if the target protein contains a S•Tag sequence).

B. Total Cell Protein (TCP) Fraction

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

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 4X SDS Sample Buffer (Cat. No. 70607-3) and sonicate with a microtip at the following settings: power level between 2–3, at 20–30% duty for 8–10 bursts (Branson Sonifier 450; sonication conditions may vary with the equipment). Alternatively, pass the sample through a 27 gauge needle several times to reduce the viscosity.
4. Immediately heat the sample for 3 min at 85°C to denature the proteins and then store at –20°C until SDS-PAGE analysis.

2X SDS Sample Buffer = 125 mM Tris-HCl, pH 6.8, 4% SDS, 5% 2-ME, or 150 mM DTT, 20% glycerol and 0.01% Bromophenol blue.

C. Medium Fraction

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. In most cases, target protein in the media is due to damage of the cell envelope rather than true secretion (Stader and Silhavy, 1990).

1. Harvest 40 ml of the culture by centrifugation at 10,000 × g for 10 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.
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 (sample concentration factor = 10X) and 100 µl of 4X SDS Sample buffer (Cat. No. 70607-3). Resuspend by vigorous vortex mixing or sonication.

   g) Immediately heat for 3 min at 85°C to denature the proteins and then store at –20°C until SDS-PAGE analysis.

   Spin filter concentration

   a) Use a low MW cut-off filter (10 kDa or lower) and follow the manufacturer's recommendations to concentrate 500 µl of medium to approximately 50 µl, yielding a concentration factor of approximately 10X.

   b) After centrifugation, determine the volume of the concentrated sample remaining, record the concentration factor on the worksheet provided and transfer to a clean tube.

   c) Rinse the spin filter membrane with hot (> 90°C) 2X SDS Sample Buffer using an amount equal in volume to that of the concentrated sample just removed from the device. Pool the 2X SDS Sample Buffer membrane rinse with the concentrated sample.

   d) Immediately heat for 3 min at 85°C to denature the proteins and then store at –20°C until SDS-PAGE analysis.

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

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D. Periplasmic Fraction

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 *Medium 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.
5. The excess supernatant (periplasmic fraction) may be removed and saved for activity assays, if desired. Save the cell pellet on ice for further processing of the soluble and insoluble cytoplasmic fractions in the following sections. Record the weight of the pellet.
6. Concentrate the periplasmic fraction by TCA precipitation or spin filtration as described in step 3 in the previous section (*Medium Sample*). Again the desired concentration factor is 10X.
7. Add an equal volume of 4X SDS Sample Buffer (Cat. No. 70607-3) and immediately heat for 3 min at 85°C to denature the proteins. Store at –20°C until SDS-PAGE analysis.

Note: The success of this procedure can be monitored with 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. Note that EDTA is not compatible with His•Bind® Resin.
3. Centrifuge at 15,000 × g for 10 min. The supernatant is the osmotic shock fraction. Analyze supernatant and pellet by SDS-PAGE.

E. Soluble Cytoplasmic Fraction

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

BugBuster and Benzonase® Nuclease treatment

BugBuster Protein Extraction Reagent is a non-mechanical method to gently disrupt the cell wall of *E. coli* resulting in the liberation of soluble protein (Grabski et al., 1999). The proprietary formulation utilizes a mixture of nonionic and zwitterionic detergents capable of cell wall perforation without denaturing soluble protein. Induced cells are harvested by centrifugation and resuspended in BugBuster. A small amount of rLysozyme™ Solution is added to increase the efficiency of cell lysis and Benzonase (a genetically engineered endonuclease) may be added to reduce lysate viscosity for increased flow rates during purification. During a brief incubation, proteins are released and nucleic acids digested. Following clarification by centrifugation, the low viscosity extract is compatible with protein assays and target protein can be directly purified with affinity chromatography resins. See Technical Bulletin 245 (BugBuster) and 261 (Benzonase) for detailed information.

Several formulations of BugBuster are available for compatibility with a variety of applications.

BugBuster Reagent	Features	Cat. No.	Size
BugBuster Protein Extraction Reagent	Tris-buffered at 1X concentration	70584-3	100 ml
		70584-4	500 ml
BugBuster HT Protein Extraction Reagent	Pre-mixed Tris-buffered BugBuster and Benzonase Nuclease at 1X concentration	70922-3	100 ml
		70922-4	500 ml
		70922-5	1 L
BugBuster 10X Protein Extraction Reagent	Dilute to 1X with choice of buffer	70921-3	10 ml
		70921-4	50 ml
		70921-5	100 ml
BugBuster (primary amine-free) Extraction Reagent	PIPPS-buffered at 1X concentration	70923-3	100 ml
		70923-4	500 ml

1. If medium and periplasmic fractions are not desired, harvest the cells from liquid media by centrifugation at 10,000 × g for 10 min using a pre-weighed centrifuge tube. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet. This will isolate the soluble cytoplasmic and periplasmic fractions.
2. Resuspend the pellet from step 5 of "*Periplasmic*" fraction or step 1 above in room temperature BugBuster by pipetting or gentle vortexing using 5 ml reagent per gram of wet cell paste. Typically, 2 ml of BugBuster is used per 50 ml of culture (a sample concentration factor of 25).
3. Add 1 KU rLysozyme Solution per 1 ml BugBuster Reagent (5 KU/g cell paste). Lysozyme addition is not necessary in pLysS or pLysE host strains.
   Optional:
   a) Add 1 µl (25 units) Benzonase Nuclease per 1 ml of BugBuster reagent used for resuspension. rLysozyme and Benzonase can be pre-mixed with BugBuster and should be used the same day stored at 4°C.
   b) Add protease inhibitors.
4. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature.
5. Centrifuge at 16,000 × g for 20 min at 4°C to remove insoluble cell debris. The pellet may be used to isolate the "*Insoluble Cytoplasmic Fraction*" page 49.
6. Transfer the supernatant to a fresh tube for analysis. Mix a sample of the supernatant with an equal volume of 4X SDS Sample Buffer (Cat. No. 70607-3). The remainder of the supernatant may be used for additional analysis or purification.
7. Immediately heat for 3 min at 85°C to denature protein and then store at –20°C until SDS-PAGE analysis.

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rLysozyme™ Solution and freeze/thaw treatment

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

1. If medium and periplasmic fractions are not desired, harvest cells from liquid culture by centrifugation at 10,000 × g for 10 min using a pre-weighed centrifuge tube. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.
2. Using the pellet from step 1 or from step 5 "*Periplasmic Fraction*", freeze the pellet completely at –20°C or –70°C.
3. Completely thaw and resuspend the frozen cell pellet by pipetting up and down or gentle vortexing in room temperature lysis buffer (50 mM Tris-HCl or NaH_2PO_4, pH 7–8, 5% glycerol, 50 mM NaCl) using 7 ml lysis buffer per gram of wet cell paste. Add protease inhibitors if necessary.

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

4. Add approximately 7.5 KU of rLysozyme per 1 ml of lysis buffer (45–60 KU/gram cell paste).
5. **Optional:** Add 1 µl (25 units) of Benzonase® Nuclease per 1 ml of lysis buffer used for resuspension. Benzonase is not recommended for nuclease free preparations. Other methods of reducing viscosity include shearing or precipitating the nucleic acids (Burgess, 1991).

Benzonase® Nuclease (Cat. No. 70664-3) degrades all forms of nucleic acids, eliminating viscosity and reducing processing time.

6. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature. The extract should not be viscous at the end of the incubation. Longer incubation time may be required if lysis is performed at 4ºC. Determine empirically.
7. Remove insoluble cell debris by centrifugation at 16,000 × g for 20 min at 4°C. The pellet may be used to isolate the "*Insoluble Cytoplasmic Fraction*" page 49.
8. Transfer the supernatant to a fresh tube for analysis and/or purification. Combine an equal volume of the supernatant with 4X SDS Sample Buffer (Cat. No. 70607-3) for SDS-PAGE Analysis.
9. Immediately heat for 3 min at 85°C to denature protein and then store at –20°C until SDS-PAGE analysis. Additional analysis and/or purification can be performed with the remainder of the soluble extract. Maintain clarified extracts on ice for short-term storage (a few hours) or freeze at –20°C until needed

Mechanical disruption

1. a) If medium and periplasmic fractions are not desired, harvest the cells from liquid media by centrifugation at 10,000 × g for 10 min. Decant and allow the pellet to drain, removing as much liquid as possible. Completely resuspend the pellet 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). Add protease inhibitors if necessary.

   or

   b) Completely resuspend the pellet from step 4 of the "*Periplasmic Fraction*" in 4 ml of cold 20 mM Tris-HCl pH 7.5 to yield a sample concentration factor of 10X (40 ml culture to 4 ml buffer volume). Add protease inhibitors if necessary.

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) S*onication*. Mix the 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.
3. Add rLysozyme™ Solution to a final concentration of 45–60 KU/gram of cell paste. Mix by pipetting up and down. Incubate at 30°C for 15 min prior to sonication. Lysozyme addition is not necessary with pLysS or pLysE host strains.

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.*

4. Centrifuge the entire lysate or a 1.5 ml sample of the lysate (for normalized SDS-PAGE analysis) at 14,000 × g for 10 min to separate the soluble and insoluble fractions.
5. For normalized SDS-PAGE analysis, transfer 100 µl of the soluble supernatant (from the 1.5 ml sample) to a new tube. Add 100 µl of 4X SDS Sample Buffer (Cat. No. 70607-3) with water. Immediately heat for 3 min at 85°C to denature proteins and then store at –20°C until SDS-PAGE analysis.
6. Remove and save the remaining supernatant for activity assays or protein purification as desired.
7. Save the insoluble pellet fraction on ice for processing, as described in the next section.

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F. Insoluble Cytoplasmic Fraction

The insoluble cytoplasmic fraction may consist of cell debris and aggregated target protein known as inclusion bodies. 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 some cases, purified inclusion bodies are suitable for direct use as antigens for the preparation of antibodies against the target protein (Harlow and Lane, 1988). Some target proteins associated with the insoluble fraction may not be in inclusion bodies. Membrane associated target proteins can pellet with the insoluble fraction and may be released into the soluble fraction by including a detergent during lysis.

Inclusion body purification after BugBuster® Reagent treatment

1. Using the insoluble pellet from the "*BugBuster and Benzonase® Nuclease isolation*" step 5, resuspend the pellet in the same volume of 1X BugBuster Reagent that was used to resuspend the original cell pellet. Pipet up and down and vortex to obtain an even suspension. Complete resuspension of the pellet is critical to obtaining a high purity preparation in order to solubilize and remove contaminating proteins.
2. Add rLysozyme™ Solution to a final concentration of 1 KU/ml. Vortex gently to mix and incubate at room temperature for 5 min.
3. Add 6 volumes of 1:10 diluted BugBuster Reagent (in deionized water) to the suspension and vortex for 1 min.
4. Centrifuge the suspension at 5,000 × g for 15 min at 4°C to collect the inclusion bodies. Remove the supernatant with a pipet.
5. Resuspend the inclusion bodies in ½ the original culture volume of 1:10 diluted BugBuster, mix by vortexing, and centrifuge as in step 5. Remove the supernatant. Repeat this step again. Resuspend once more but centrifuge at 16,000 × g for 15 min at 4°C and remove the supernatant.
6. a) For normalized SDS-PAGE analysis, resuspend the final pellet in 1.5 ml 1% SDS Sample Buffer with heating and vigorous mixing or sonication if necessary (resuspension in this volume maintains the concentration factor at 10X). Remove a 100 µl sample and combine with 100 µl of 4X SDS Sample Buffer (Cat. No. 70607-3). Immediately heat for 3 min at 85°C to denature proteins and then store at –20°C until SDS-PAGE analysis.
   b) For purification, resuspend the pellet in the denaturing buffer of your choice, preferably using a buffer compatible with the desired purification method. The final pellet of inclusion bodies is compatible with resuspension in 1X Solubilization Buffer provided in Novagen's Protein Refolding Kit (see Technical Bulletin 234) or other denaturing buffers.

Inclusion body purification after mechanical cell lysis

For normalized SDS-PAGE analysis

1. Wash the insoluble pellet by resuspending in 750 µl (from a 40 ml culture) 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. Remove a 100 µl sample and combine with 100 µl of 4X SDS Sample Buffer (Cat. No. 70607-3). Immediately heat for 3 min at 85°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

For protein purification preparation

1. Wash the insoluble pellet by resuspending in 16 ml (per 40 ml culture volume) of appropriate buffer (without denaturant) for the purification resin.
2. Sonicate briefly to resuspend the pellet thoroughly and shear DNA.
3. Centrifuge at 5,000 × g for 15 min to collect the inclusion bodies and cellular debris while leaving other proteins in solution.

4. Remove the supernatant and resuspend the pellet in 20 ml buffer (per 40 ml culture volume) without denaturant. Repeat Step 3. Sonication may be necessary to resuspend the pellet. Sometimes repeating this step several times releases more trapped proteins.
5. Remove the supernatant from the final centrifugation and resuspend the pellet in 5 ml buffer containing a denaturant to solubilize the inclusion bodies, preferably using a buffer compatible with the desired purification method. The final pellet is compatible with resuspension in 1X Solubilization Buffer provided in Novagen's Protein Refolding Kit (see Technical Bulletin 234) or other denaturing buffers.
6. Full or partially denatured inclusion bodies may be directly purified using His•Tag® fusion proteins (Technical Bulletin 054) or S•Tag™ fusion proteins (Technical Bulletin 160, 087). Denatured proteins may be refolded prior to affinity purification (see Technical Bulletin 234). In some cases, incubating the sample on ice up to 1 hour may help to solubilize the inclusion bodies.

G. Preparation of Extracts with PopCulture® Reagent

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

An induced culture of *E. coli* is treated with PopCulture Reagent for 10–15 min at room temperature in the presence of rLysozyme; additional treatment with Benzonase Nuclease is optional. The PopCulture extract can also be used directly in protein assays such as FRETWorks™ S•Tag™, S•Tag Rapid or GST•Tag™ Assay Kits. This prepared extract can be directly combined with an equilibrated chromatography resins (e.g. GST•Mag™, His•Mag™) for purification. PopCulture Reagent is also available in the RoboPop™ Purification Kits that include 96-well culture and collection plates for high throughput processing of 1 ml cultures of His•Tag or GST•Tag fusion proteins (see Technical Bulletin 327).

1. Add 0.1 culture volume of PopCulture Reagent to an induced culture.
2. Add 40 U (1 µl of a 1:750 dilution) rLysozyme™ Solution per 1 ml of original culture volume. Additional lysozyme is not necessary if the host strain contains the pLysS or pLysE plasmid producing lysozyme.
   Optional:
   Add 1 µl (25 U) Benzonase® Nuclease per 1 ml of the original culture volume. rLysozyme and Benzonase can be pre-mixed with PopCulture and should be used the same day stored at 4°C.
3. Pipet up and down to mix and incubate for 10–15 min at room temperature.
4. This prepared extract can be assayed directly or combined with an equilibrated chromatography resin.

SDS-PAGE of total cell protein: Combine an aliquot of the prepared extract with an equal volume 4X SDS Sample Buffer (Cat. No. 70607-3) for detection with Coomassie staining or Western blotting. Given an OD between 3–5, a highly expressed protein may be detected with Coomassie staining using a 10 µl sample. Low expression levels and cell densities may require Western blotting.
Quantitative assay: Fusion tag specific quantitative assays such as FRETWorks™ S•Tag™ Assay, S•Tag Rapid Assay and GST•Tag™ Assay are compatible with the PopCulture extracts as are Bradford and BCA protein assays.
Target protein specific assays: Because proteins generally retain their activities and conformation, protein specific activity and immunoassays are likely to be compatible with PopCulture extraction.
Purification: PopCulture is Tris-buffered and is compatible with both Tris (His•Bind®) and phosphate (GST•Bind™) buffer purification systems at a neutral pH. The use of His•Mag™ or GST•Mag™ Agarose Beads enables the entire procedure (culture through purification) to be carried out in a single tube without columns or centrifugation. RoboPop™ Kits provide 96-well culturing and purification plates with PopCulture and purification resins for His•Tag or GST•Tag fusion proteins. (Technical Bulletins 327). PopCulture is expected to be compatible with many other affinity purification resins.

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VII. Detecting and Quantifying Target Proteins

Protein expression can be rapidly determined by SDS-PAGE analysis of cell extracts followed by staining with Coomassie blue, which in many cases will reveal the target protein as a unique band when run adjacent to an uninduced extract. Western blotting is a more specific and sensitive method for identification and estimation of expression levels, and can be conveniently performed using fusion tag-specific reagents or protein-specific antibodies or other ligands. Activity assays are protein-dependent, especially in crude extracts, and are often performed following some degree of purification. However, crude extracts prepared with PopCulture™ Reagent can be immediately assayed (see *PopCulture Quick Screen*, page 43).

A. Normalized SDS-PAGE Gel Loading

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) or Trail Mix™ Markers (Cat. No. 70980-3) provide accurate size references for proteins between 10 kDa and 225 kDa on Coomassie blue stained gels.

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. The sample concentration factor represents the volume of original culture used to produce the fraction divided by the final volume of the fraction. For example, if 1 ml of culture is used to prepare the fraction and after processing the final volume is 100 µl, then the sample concentration factor is 10. If larger gels are used, the loading volumes should be scaled up accordingly. The loading volume of each sample will need to be calculated, because the actual concentration factor for a given fraction may vary.

				Volume to Load	
				15-well mini-gel	**10-well mini-gel**
	Sample conc. factor	**OD_{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					

B. Detection/Assay Tools for Fusion Tags

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

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

Western Blotting	Cat. No.	Size	Technical Bulletin No./Application
CBD•Tag™ detection			
CBD_{clos}•Tag Antibody	70119-3	25 µl	Polyclonal, WB
CBD_{cenA}•Tag Antibody	70157-3	500 µl	Polyclonal, WB
CBD_{cex}•Tag Antibody	70158-3	500 µl	Polyclonal, WB
GST•Tag™ detection			
GST•Tag Monoclonal Antibody	71097-3	50 µg 250 µg	TB325 IF, IP, QA, WB
His•Tag® detection			
His•Tag Monoclonal Antibody	70796-4 70796-3	3 µg 100 µg	TB283 IF, IP, WB
His•Tag AP Western Reagents	70972-3	25 blots	TB283 colorimetric detection
His•Tag AP LumiBlot™ Reagents	70973-3	25 blots	TB283 chemiluminescent detection
His•Tag HRP LumiBlot Reagents	70974-3	25 blots	TB283 chemiluminescent detection
HSV•Tag® detection			
HSV•Tag Monoclonal Antibody	69171-3 69171-4	40 µg 200 µg	TB067 WB
Nus•Tag™ detection/assay			
Nus•Tag Monoclonal Antibody	71127-3 71127-4	50 µg 250 µg	TB328 WB
S•Tag™ detection/assay			
S-protein AP Conjugate	69598-3	50 µl	TB097 WB
S-protein HRP Conjugate	69047-3	50 µl	TB136 WB
Biotinylated S-protein	69218-3	250 µl	WB
S-protein FITC Conjugate	69060-3	200 µl	TB143 IF
S•Tag AP Western Blot Kit	69213-3	25 blots	TB082 colorimetric detection
S•Tag AP LumiBlot Kit	69099-3	25 blots	TB164 chemiluminescent detection
S•Tag HRP LumiBlot Kit	69058-3	25 blots	TB145 chemiluminescent detection
T7•Tag® detection			
T7•Tag Monoclonal Antibody	69522-3 69522-4	50 µg 250 µg	TB015 IF, IP, WB
Biotinylated T7•Tag Antibody	69968-3	125 µl	TB106 WB
T7•Tag Antibody HRP Conjugate	69048-3	100 µl	TB137 WB
T7•Tag Antibody AP Conjugate	69999-3	50 µl	TB112 WB
T7•Tag AP LumiBlot Kit	70237-3	25 blots	TB212 chemiluminescent detection
T7•Tag HRP LumiBlot Kit	70238-3	25 blots	TB213 chemiluminescent detection

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

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Quantitative Assay	Cat. No.	Size	Technical Bulletin No./Sensitivity
FRETWorks™ S•Tag™ Assay Kit	70724-3 70724-4	100 assays 1000 assays	TB251; fluorescent assay, Limit < 1 fmol
S•Tag Rapid Assay Kit	69212-3	100 assays	TB082; Limit 20 fmol
GST•Tag™ Assay Kit	70532-3	100 assays	TB236; colorimetric assay, Limit 8 pmol

Western Blot Protein Markers	Cat. No	Size	Technical Bulletin No./ Size Standards
Perfect Protein™ Western Markers	69959-3	25 lanes	TB102; 15, 25, 35, 50, 75, 100 and 150 kDa
Trail Mix™ Western Markers	70982-3	25 lanes	TB310; 15, 25, 35, 50, 75, 100 and 150 kDa, and 15, 16, 100 kDa prestained markers

VIII. 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. Many approaches can be used to purify target proteins expressed with the pET System. One advantage of the system is that in many cases the target protein accumulates to such high levels that it constitutes a high percentage of the total cell protein. Therefore, it is relatively straightforward to isolate the protein in two or three chromatographic steps by conventional methods (ion exchange, gel filtration, etc.).

A variety of affinity purification methods are available that take advantage of the various peptide fusion tags available with pET vectors. In many cases, the use of an affinity method enables the purification of the target protein to near homogeneity in one step. Purification may include cleavage of part or all of the fusion tag with enterokinase, Factor Xa, or thrombin proteases. 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 using the methods described in *Target Protein Verificatio*n, pages 42–50. 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.

A. Purification Tools

A brief description of products for extract preparation and affinity chromatography is indicated below. For detailed information, see the indicated Technical Bulletin available at www.novagen.com

Extraction Reagents	Cat. No.	Size	Technical Bulletin No./Capacity and Features
BugBuster® Protein Extraction Reagent	70584-3 70584-4	100 ml 500 ml	TB245 Use 5 ml/g wet cell paste. Tris-buffered.
BugBuster HT Protein Extraction Reagent	70922-3 70922-4	100 ml 500 ml	TB245 Use 5 ml/g wet cell paste. Tris-buffered and pre-mixed with Benzonase Nuclease.
BugBuster 10X Protein Extraction Reagent	70921-3 70921-4 70921-5	10 ml 50 ml 100 ml	TB245 Dilute to 1X with choice of buffer and use 5 ml/g wet cell paste.
BugBuster (primary amine-free) Extraction Reagent	70923-3 70923-4	100 ml 500 ml	TB245 Use 5 ml/g wet cell paste. PIPPS-buffered.
PopCulture® Reagent	71092-3 71092-4 71092-5	15 ml 75 ml 250 ml	TB323 Use 0.1 volume per ml of culture.
rLysozyme™ Solution	71110-3 71110-4 71110-5	300 KU 1200 KU 6000 KU	TB334 and TB323 Use 40 U per 1 ml of culture volume with PopCulture Reagent and 1 KU per 1 ml BugBuster Reagent.
Benzonase® Nuclease, Purity > 90%	70746-3 70746-4	10,000 U 2,500 U	TB245, 323, 261 Use 25 U per ml original culture volume with PopCulture and BugBuster Reagent
CBD•Tag™ purification	**Cat. No.**	**Size**	**Technical Bulletin No./Capacity and Features**
CBIND™ 100 Resin	70120-3	25 g	TB189 Capacity is 40 mg/g resin
CBIND 200 Resin	70121-3	25 g	TB189 Capacity is 5 mg/g resin
CBIND 300 Cartridges	70124-3 70124-4	pkg/10 pkg/50	TB189 Pre-packed, Capacity is 1.5 mg/cartridge
CBIND 900 Cartridges	70132-3 70132-4	pkg/10 pkg/50	TB189 Pre-packed, Capacity is 4.5 mg/cartridge
CBIND ReadyRun Columns	70144-3 70144-4	pkg/12 pkg/60	TB189 Pre-packed, Capacity is 10 mg per column.
GST•Tag™ purification	**Cat. No.**	**Size**	**Technical Bulletin No./Capacity and Features**
GST•Bind™ Resin	70541-3 70541-4	10 ml 50 ml	TB235 Capacity is 5–8 mg/ml settled resin
GST•Bind Buffer Kit	70534-3		TB235 All buffers for ten 2.5 ml columns
GST•Mag™ Agarose Beads	71084-3 71084-4	2 × 1 ml 10 × 1 ml	TB235 Magnetic agarose beads. Capacity up to 2 mg/ml settled volume
BugBuster GST•Bind Purification Kit	70794-3		TB235 GST•Bind Resin and Buffer, BugBuster, Benzonase and Chromatography Columns.
PopCulture GST•Mag Purification Kit	71113-3		TB235 Process 40 × 3 ml cultures purifying up to 150 µg per 3 ml culture.
RoboPop™ GST•Mag Purification Kit	71102-3		TB327 Purify up to 4.8 mg per 96 wells
His•Tag® purification	**Cat. No.**	**Size**	**Technical Bulletin No./Capacity and Features**
Ni-NTA His•Bind® Resin	70666-3 70666-4 70666-5	10 ml 25 ml 100 ml	TB273 Capacity is 5–10 mg/ml settled resin
Ni-NTA Superflow	70691-3 70691-4 70691-5	10 ml 25 ml 100 ml	TB273 Capacity is 5–10 mg/ml settled resin, high flow rates and pressures
Ni-NTA Buffer Kit	70899-3		TB273 All buffers for native purification using Ni-NTA His•Bind and Ni-NTA Superflow resins.

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His•Bind® Resin	69670-3 69670-4 69670-5	10ml 50 ml 100ml	TB054 Capacity is 8 mg/ml settled resin
His•Bind Buffer Kit	69755-3		TB054 All buffers for native purification using His•Bind Resin
His•Bind Columns	70971-3 70971-4	pkg/5 pkg/25	TB054 pre-packed, pre-charged; Capacity is 10 mg per column.
His•Bind Quick Columns	70159-3 70159-4	pkg/12 pkg/60	TB054 pre-packed, pre-charged, requires vacuum, Capacity is 5 mg per column.
His•Bind Quick 300 Cartridges	70155-3 70155-4	pkg/10 pkg/50	TB054 pre-packed, pre-charged, Capacity is 0.5 mg per cartridge
His•Bind Quick 900 Cartridges	70153-3 70153-4	pkg/10 pkg/50	TB054 pre-packed, pre-charged, Capacity is 2 mg per cartridge
His•Mag™ Agarose Beads	71002-3 71002-4	2 ml 10 ml	TB054 magnetic agarose beads, pre-charged, Capacity is 5 mg per ml settled beads.
His•Bind Quick Buffer Kit	70665-3		TB054 all buffers for native purification using His•Bind Columns, Quick Columns, Cartridges and His•Mag Agarose Beads. No charge buffer included.
His•Bind Purification Kit	70239-3		TB054 10 ml His•Bind Resin, Buffers and Chromatography Columns
BugBuster® Ni-NTA His•Bind Purification Kit	70751-3		TB273 10 ml Ni-NTA His•Bind Resin, BugBuster, Benzonase and Chromatography Columns.
BugBuster His•Bind Purification Kit	70793-3		TB054 10 ml His•Bind Resin and Buffer, BugBuster, Benzonase and Chromatography Columns.
PopCulture® His•Mag™ Purification Kit	71114-3		TB054 Process 40 × 3 ml cultures purifying up to 375 µg per 3 ml culture.
RoboPop™ His•Mag Purification Kit	71103-3		TB327 Purify up to 12 mg per 96 wells
S•Tag™ purification	**Cat. No.**	**Size**	**Technical Bulletin No./Capacity and Features**
S-protein Agarose	69704-3 69704-4	2 ml 5 × 2 ml	TB087, TB160 Purify up to 1 mg per 2 ml settled resin
S•Tag Thrombin Purification Kit	69232-3		TB087 Purify and cleave up to 1 mg target protein per kit (2 ml settled resin)
S•Tag rEK Purification Kit	69065-3		TB160 Purify and cleave up to 1 mg target protein per kit (2 ml settled resin)
T7•Tag® purification	**Cat. No.**	**Size**	**Technical Bulletin No./Capacity and Features**
T•Tag Antibody Agarose	69026-3	2 × 1 ml	TB125 minimum 300 µg T7•Tag β-galactosidase per ml settled resin. May vary between target proteins.
T•Tag Affinity Purification Kit	69025-3		TB125 minimum 300 µg T7•Tag β-galactosidase per ml settled resin. May vary between target proteins. One ml of agarose, buffers and columns for purification.

Proteases and Cleavage Capture Kits	**Cat. No.**	**Size**	**Technical Bulletin No./Description**
Thrombin, Restriction Grade	69671-3	50 U	TB188
Biotinylated Thrombin	69672-3	50 U	TB188
Thrombin Cleavage Capture Kit	69022-3		TB188 50 U Biotinylated Thrombin and Streptavidin Agarose for Biotinylated Thrombin removal
Recombinant Enterokinase	69066-3	50 U	TB150
Enterokinase Cleavage Capture Kit	69067-3		TB150 50 U Recombinant Enterokinase and EKapture™ Agarose for enterokinase removal
Factor Xa, Restriction Grade	69036-3	400 U	TB205
Factor Xa Cleavage Kit	69037-3		TB205 400 U Factor Xa and Xarrest™ Agarose for Factor Xa removal

B. Solubilization and Refolding Proteins

A variety of methods have been published describing refolding of insoluble proteins (Marston and Hartley, 1990; Kurucz et al., 1995; Burgess, 1996; Frankel and Leinwand, 1996; Rudolph and Lilie, 1996; Mukhopadhyay, 1997). 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 Technical Bulletin 234, available at www.novagen.com.

Depending on the target protein, expression conditions and intended application, proteins solubilized from washed inclusion bodies may be > 90% homogeneous and may not require further purification. Purification under fully denaturing conditions (prior to refolding) is possible using His•Tag® fusion proteins and His•Bind® metal chelation chromatography (see Technical Bulletin 054). 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 Technical Bulletin 160 or Technical Bulletin 087). 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™, and T7•Tag®).

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IX. 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 page 19.

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

The BetaRed™ (Cat. No. 70978-3) β-Galactosidase Assay Kit provides a rapid, sensitive measurement of β-galactosidase activity in cell extracts. Extracts prepared with BugBuster® Reagent, PopCulture™ Reagent, or standard PBS and Tris-based lysis buffers are compatible with this assay as well as rLysozyme™ Solution and Benzonase® Nuclease. The colorimetric BetaRed Assay is 10-fold more sensitive (1 pg) than ONPG-based assays. For a detailed protocol, see Technical Bulletin 303.

X. Acknowledgments

Novagen gratefully acknowledges Bill Studier, Alan Rosenberg, and John Dunn of Brookhaven National Laboratories for many helpful discussions and for their permission to print portions of the sections *About the System* and *Optimizing Expression*.

XI. References

Aslanidis, C. and de Jong, P.J. (1990) *Nucl. Acids Res.* **18**, 6069–6074.

Aslund, F., Bessette, P.H., Georgiou, G. and Beckwith, J. (1999) *in**Nova**tions* **10**, 11–12.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K (1989) *in* Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Bessette, P.H., Aslund, F., Beckwith, J. and Georgiou, G. (1999) *Proc. Natl. Acad. Sci.* **96**, 13703–13708.

Brennan, W.A. and Lin, S.H. (1996) *in* " Strategies for Protein Purification and Characterization, A Laboratory Manual," Cold Spring Harbor Laboratory Press.

Brinkmann, U., Mattes, R.E. and Buckel, P. (1989) *Gene* **85**, 109–114.

Burgess, R.R. (1991) *Meth. Enzymol.* **208**, 3–10.

Burgess, R.R. (1996) *Meth. Enzymol.* **273**, 145–149.

Buttistuzzi, G., Esan, G.J.F., Fasuan, F.A., Modiano, G. and Luzzatto, L. (1977) *Am. J. Hum. Genet.* **29**, 31–36.

Calderone, T.L., Stevens, R.D. and Oas, T.G. (1996) *J. Mol. Biol.* **262**, 407–412.

Chang, A.C.Y. and Cohen, S.N. (1978) *J. Bacteriol.* **134**, 1141.

Chang, J.Y. (1985) *Eur. J. Biochem.* **151**, 217–224.

Chen, G.F. and Inouye, M. (1990) *Nucl. Acids Res.* **18**, 1465–1473.

Collins-Racie, L.A., McColgan, J.M., Grant, K.L., DiBlasio-Smith, E.A., McCoy, J.M. and LaVallie, E.R. (1995) *Biotechnology (N Y)* **13**, 982–987.

Davis, G.D., Elisee, C., Newham, D.M. and Harrison, R.G. (1999) *Biotechnol. Bioeng.* **65**, 382–388.

Del Tito, B.J., Jr., Ward, J.M., Hodgson, J., Gershater, C.J., Edwards, H., Wysocki, L.A., Watson, F.A., Sathe, G. and Kane, J.F. (1995) *J. Bacteriol.* **177**, 7086–7091.

Doherty, A.J., Ashford, S.R., Brannigan, J.A., and Wigley, D. B. (1995) *Nucl. Acids Res.* **23**, 2074–2075.

Dubendorff, J.W. and Studier, F.W. (1991) *J. Mol. Biol.* **219**, 45–59.

Dunn, J.J. and Studier, F.W. (1983) *J. Mol. Biol.* **166**, 477–535.

Eron, L. and Block, R. (1971) *Proc. Natl. Acad. Sci.* **68**, 1828–1832.

Fourrier, K. and Hayes, S. (2001) *in**Nova**tions* **12**, 8–9.

Frankel, S.A. and Leinwand, L.A. (1996) *U.S. patent no. 5,240,834*.

Fried, M.G. and Crothers, D.M. (1984) *J. Mol. Biol.* **172**, 241–262.

Grabski, A., Drott, D., Handley, M., Mehler, M. and Novy, R. (2001) *in**Nova**tions* **13**, 1–4.

Grabski, A., McCormick, M. and Mierendorf, R. (1999) *in**Nova**tions* **10**, 17–19.

Grabski, A., Mehler, M., Drott, D. and Van Dinther, J. (2002) *in**Nova**tions* **14**, 2–5.

Grodberg, J. and Dunn, J.J. (1988) *J. Bacteriol.* **170**, 1245–1253.

Grossman, T.H., Kawasaki, E.S., Punreddy, S.R. and Osburne, M.S. (1998) *Gene* **209**, 95–103.

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Halling, S.M. and Smith, S. (1985) *Bio/Technology* **3**, 715–720.

Harlow, E. and Lane, D. (1988) *Antibodies, A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Harrison, R.G. (2000) *in**Nova**tions* **11**, 4–7.

Haun, R.S., Serventi, I.M. and Moss, J. (1992) *Biotechniques* **13**, 515–518.

Hirel, P.H., Schmitter, M.J., Dessen, P., Fayat, G. and Blanquet, S. (1989) *Proc. Natl. Acad. Sci.* **86**, 8247–8251.

Hua, Z., Wang, H., Chen, D., Chen, Y. and Zhu, D. (1994) *Biochem. Mol. Biol. Int.* **32**, 537–543.

Huang, J., Villemain, J., Padilla, R. and Sousa, R. (1999) *J. Mol. Biol.* **293**, 457–475.

Inouye, M., Arnheim, N. and Sternglanz, R. (1973) *J. Biol. Chem.* **248**, 7247.

Joly, J.C. and Swartz, J.R. (1997) *Biochemistry* **36**, 10067–10072.

Kajava, A.V., Zolov, S.N., Kalinin, A.E. and Nesmeyanova, M.A. (2000) *J. Bacteriol.* **182**, 2163–2169.

Kane, J.F. (1995) *Curr. Opin. Biotechnol.* **6**, 494–500.

Kelley, K.C., Huestis, K.J., Austen D.A., Sanderson, C.T., Donoghue, M.A. Stickel, S. K, Kawasaki, E.S., and Osburne M.S. (1995) *Gene* **156**, 33–36.

Kurland, C. and Gallant, J. (1996) *Curr. Opin. Biotechnol.* **7**, 489–493.

Kurucz, I., Titus, J.A., Jost, C.R. and Segal, D.M. (1995) *Mol. Immunol.* **32**, 1443–1452.

Lathrop, B.K., Burack, W.R., Biltonen, R.L. and Rule, G.S. (1992) *Protein Expr. Purif.* **3**, 512–517.

LaVallie, E.R., DiBlasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F. and McCoy, J.M. (1993) *Bio/Technology* **11**, 187–193.

Le Bonniec, B.F., MacGillivray, R.T. and Esmon, C. T. (1991) *J. Bio. Chem.* **266**, 13,706–13,803.

Le Bonniec, B.F., Myles, T., Johnson, T., Knight, C.G. Tapparello, C. and Stone S. R. (1996) *Biochem.* **35**, 7114–7122.

Leahy, D.J.H., W.A., Aukhil, I. and Erickson, H.P. (1992) *Science* **258**, 987–991.

Lee, N., Zhang, S.Q., Cozzitorto, J., Yang, J.S. and Testa, D. (1987) *Gene* **58**, 77–86.

Looman, A.C., Bodlaender, J., de Gruyter, M., Vogelaar, A. and van Knippenberg, P.H. (1986) *Nucl. Acids Res.* **14**, 5481–5497.

Marston, F.A. and Hartley, D.L. (1990) *Meth. Enzymol.* **182**, 264–276.

McAllister, W.T., Morris, C., Rosenberg, A.H. and Studier, F.W. (1981) *J. Mol. Biol.* **153**, 527.

McCormick, M., Yaeger, K., Novy, R. and Van Oosbree, T. (1994) *in**Nova**tions* **2**, 7.

McCormick, M.R. and Ambuel, Y. (2000) *In**Nova**tions* **11**, 8–11.

Mierendorf, R., Yaeger, K. and Novy, R. (1994) *in**Nova**tions* **1**, 1–3.

Missiakas, D., Georgopoulos, C. and Raina, S. (1994) *EMBO J.* **13**, 2013–2020.

Moffatt, B.A. and Studier, F.W. (1987) *Cell* **49**, 221.

Mukhopadhyay, A. (1997) *Adv. Biochem. Eng. Biotechnol.* **56**, 61–109.

Novy, R., Berg, J., Yaeger, K. and Mierendorf, R. (1995) *in**Nova**tions* **3**, 7–9.

Novy, R., Drott, D., Yaeger, K. and Mierendorf, R. (2001) *in**Nova**tions* **12**, 1–3.

Novy, R. and Morris, B. (2001) *in**Nova**tions* **13**, 8–10.

Novy, R., Yaeger, K. and Miller, S.A. (1997) *in**Nova**tions* **7**, 4–7.

Pan, S. and Malcom, B.A. (2000) *Biotechniques* **29**, 1234–1238.

Preibisch, G., Ishihara, H., Tripier, D. and Leineweber, M. (1988) *Gene* **72**, 179–186.

Prinz, W.A., Aslund, F., Holmgren, A. and Beckwith, J. (1997) *J. Biol. Chem.* **272**, 15661–15667.

Raina, S. and Missiakas, D. (1997) *Annu. Rev. Microbiol.* **51**, 179–202.

Rietsch, A., Belin, D., Martin, N. and Beckwith, J. (1996) *Proc. Natl. Acad. Sci.* **93**, 13048–13053.

Ritz, D., Lim, J., Reynolds, C.M., Poole, L.B. and Beckwith, J. (2001) *Science* **294**, 158–160.

Rosenberg, A.H., Goldman, E., Dunn, J.J., Studier, F.W. and Zubay, G. (1993) *J. Bacteriol.* **175**, 716–722.

Rosenberg, A.H., Lade, B.N., Chui, D.S., Lin, S.W., Dunn, J.J. and Studier, F.W. (1987) *Gene* **56**, 125–135.

Rudolph, R. and Lilie, H. (1996) *FASEB J.* **10**, 49–56.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schein, C.H. and Noteborn, M.H.M. (1989) *Bio/Technology* **7**, 1141–1148.

Schenborn, E.T. and Mierendorf, R. (1985) *Nucleic Acids Res* **13**, 6223-6236.

Schenk, P.M., Baumann, S., Mattes, R. and Steinbiss, H.H. (1995) *Biotechniques* **19**, 196–200.

Seidel, H.M., Pompliano, D.L. and Knowles, J.R. (1992) *Biochemistry* **31**, 2598–2608.

Sektas, M. and Szybalski, W. (2002) *in**Nova**tions* **14**, 6–8.

Snyder, W.B. and Silhavy, T.J. (1995) *J. Bacteriol.* **177**, 953–963.

Sone, M., Akiyama, Y. and Ito, K. (1997) *J. Biol. Chem.* **272**, 10349–10352.

Sorensen, M.A., Kurland, C.G. and Pedersen, S. (1989) *J. Mol. Biol.* **207**, 365–377.

Stader, J.A. and Silhavy, T.J. (1990) *Meth. Enzymol.* **185**, 166–187.

Stewart, E.J., Aslund, F. and Beckwith, J. (1998) *EMBO J.* **17**.

Studier, F.W. (1991) *J. Mol. Biol.* **219**, 37–44.

Studier, F.W. and Moffatt, B.A. (1986) *J. Mol. Biol.* **189**, 113–130.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) *Meth. Enzymol.* **185**, 60–89.

Tessier, L.H., Sondermeyer, P., Faure, T., Dreyer, D., Benavente, A., Villeval, D., Courtney, M. and Lecocq, J.P. (1984) *Nucleic Acids Res.* **12**, 7663–7675.

Tobias, J.W., Shrader, T.E., Rocap, G. and Varshavsky, A. (1991) *Science* **254**, 1374–1377.

Wickner, W., Driessen, A.J.M. and Hartl, F.-U. (1991) *Annu. Rev. Biochem.* **60**, 101–124.

Wood, W. (1966) *J. Mol. Biol.* **16**, 118–133.

Zahn, K. (1996) *J. Bacteriol.* **178**, 2926–2933.

Zapun, A., Missiakas, D., Raina, S. and Creighton, T.E. (1995) *Biochemistry* **34**, 5075–5089.

Zhang, S.P., Zubay, G. and Goldman, E. (1991) *Gene* **105**, 61–72.

Zhang, X. and Studier, F.W. (1997) *J. Mol. Biol.* **269**, 10–27.

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XIII. 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: (631) 344-7134. Contact: Christine Brakel.
2. No materials that contain the cloned gene for T7 RNA polymerase may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this assurance letter and agrees to be bound by its terms. This limitation applies to any of the following materials that are included in this kit and to any derivatives you may make of them:

E. coli AD494(DE3)	*E. coli* Origami(DE3)pLacI
E. coli AD494(DE3)pLysS	*E. coli* Origami B-(DE3)
E. coli B834(DE3)	*E. coli* Origami B(DE3)pLysS
E. coli B834(DE3)pLysS	*E. coli* Origami B(DE3)pLacI
E. coli BL21(DE3)	*E. coli* Rosetta(DE3)
E. coli BL21(DE3)pLysS	*E. coli* Rosetta(DE3)pLysS
E. coli BL21(DE3)pLysE	*E. coli* Rosetta(DE3)pLacI
E. coli BL26(DE3)pLysE	*E. coli* Rosetta-gami(DE3)
E. coli BL21*trxB*(DE3)	*E. coli* Rosetta-gami(DE3)pLysS
E. coli BL21*trxB*(DE3)pLysS	*E. coli* Rosetta-gami(DE3)pLacI
E. coli BLR(DE3)	*E. coli* RosettaBlue(DE3)
E. coli BLR(DE3)pLysS	*E. coli* RosettaBlue(DE3)pLysS
E. coli HMS174(DE3)	*E. coli* RosettaBlue(DE3)pLacI
E. coli HMS174(DE3)pLysS	*E. coli* Tuner(DE3)
E. coli HMS174(DE3)pLysE	*E. coli* Tuner(DE3)pLysS
E. coli NovaBlue(DE3)	*E. coli* Tuner(DE3)pLacI
E. coli Origami(DE3)	Bacteriophage λCE6
E. coli Origami(DE3)pLysS	Bacteriophage λCE6

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.

XIV. Bacterial Strain Non-Distribution Agreement

By purchase of the AD494, BL21*trxB*, Origami, Origami B, Rosetta, RosettaBlue or Rosetta-gami host strains and acceptance of the following terms, Novagen grants a limited license to use the AD494, BL21*trxB*, Origami, or Origami B, Rosetta, RosettaBlue, and Rosetta-gami host strains for the cloning and expression of genes. The intent of this license is not to limit the research use of these materials, but to protect against unauthorized commercial distribution of the strains by third parties.

1. The AD494, BL21*trxB*, Origami, Origami B, Rosetta, RosettaBlue, Rosetta-gami host strains or any derivative therefrom is not to be offered for resale or distributed outside your laboratory.
2. Gene clones and libraries in the AD494, BL21*trxB*, Origami, Origami B, Rosetta, RosettaBlue, and Rosetta-gami host strains may be distributed for research purposes only, provided that the recipient acknowledge the foregoing condition.

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.

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XV. Appendix: pET System Host Strains and Lambda Phages

λDE3 Lysogens for Protein Expression

The pET expression hosts are lysogens of bacteriophage λDE3, as indicated by the (DE3) in their names and are suitable for production of protein from target genes cloned in pET vectors. The pLysS and pLysE designation is given to hosts carrying a pET-compatible plasmid that encodes T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase. These strains are used to supress basal expression of T7 RNA polymerase prior to induction and thus stabilize pET recombinants encoding target proteins that affect cell growth and viability.

Strain	Size	Competent Cells Cat. No.	Glycerol Stock Cat. No.	Application
AD494(DE3)	0.2 ml		69020-3	Allows disulfide bond formation in *E. coli* cytoplasm.
	0.4 ml	69013-3		
	1.0 ml	69013-4		
AD494(DE3)pLysS	0.2 ml		69021-3	
	0.4 ml	69014-3		
	1.0 ml	69014-4		
B834(DE3)	0.2 ml		69288-3	Selenomethionine and ^{35}-S-met labeling
	0.4 ml	69041-3		
	1.0 ml	69041-4		
B834(DE3)pLysS	0.2 ml		69289-3	
	0.4 ml	69042-3		
	1.0 ml	69042-4		
BL21(DE3)	0.2 ml		69387-3	Protects target protein from *lon* and *ompT* proteases
	0.4 ml	69450-3		
	1.0 ml	69450-4		
HT96™ BL21(DE3)	1 plate	71012-3		
	4 plates	71012-4		
	20 plates	71012-5		
BL21(DE3) Singles™	11 rxn	70235-3		
	22 rxn	70235-4		
BL21(DE3)pLysS	0.2 ml		69388-3	
	0.4 ml	69451-3		
	1.0 ml	69451-4		
BL21(DE3)pLysS Singles	11 rxn	70236-3		
	22 rxn	70236-4		
Bl21(DE3)pLysE	0.2 ml		69389-3	
BL21*trxB*(DE3)	0.2 ml		70506-3	Allows disulfide bond formation in *E. coli* cytoplasm and protects target protein from *lon* and *ompT* proteases
	0.4 ml	70508-3		
	1.0 ml	70508-4		
BL21*trxB*(DE3)pLysS	0.2 ml		70507-3	
	0.4 ml	70509-3		
	1.0 ml	70509-4		
BLR(DE3)	0.2 ml		69208-3	A *recA*$^-$ derivative of BL21 that may stabilize target plasmids containing repetitive sequences.
	0.4 ml	69053-3		
	1.0 ml	69053-4		
BLR(DE3)pLysS	0.2 ml		69209-3	
	0.4 ml	69956-3		
	1.0 ml	69956-4		
HMS174(DE3)	0.2 ml		69381-3	A K-12 *recA*$^-$ strain that may stabilize target plasmids containing repetitive sequences
	0.4 ml	69453-3		
	1.0 ml	69453-4		
HMS174(DE3)pLysS	0.2 ml		69382-3	
	0.4 ml	69454-3		
	1.0 ml	69454-4		
HMS174(DE3)pLysE	0.2 ml		69393-3	

Strain	Size	Competent Cells Cat. No.	Glycerol Stock Cat. No.	Application
NovaBlue(DE3)	0.4 ml	69284-3		High stringency host with *recA⁻ endA1 and -* *lacI*^{q} mutations. Recommended for use with the NovaTope™ System
	1.0 ml	69284-4		
Origami™(DE3)	0.2 ml		70617-3	Allows disulfide bond formation in *E. coli* cytoplasm.
	0.4 ml	70627-3		
	1.0 ml	70627-4		
Origami(DE3) Singles	11 rxn	70630-3		
	22 rxn	70630-4		
Origami(DE3)pLysS	0.2 ml		70618-3	
	0.4 ml	70628-3		
	1.0 ml	70628-4		
Origami(DE3)pLysS Singles	11 rxn	70631-3		
	22 rxn	70631-4		
Origami B(DE3)	0.2 ml		70830-3	Allows disulfide bond formation in *E. coli* cytoplasm and protects target protein from *lon* and *ompT* proteases. A derivative of Tuner™ (*lacY1*) enabling low levels expression in all cells for solubility and activity enhancement.
	0.4 ml	70837-3		
	1.0 ml	70837-4		
Origami B(DE3)pLysS	0.2 ml		70832-3	
	0.4 ml	70839-3		
	1.0 ml	70839-4		
Rosetta™(DE3)	0.2 ml		70950-3	Enhances expression of proteins that contain codons rarely used in *E. coli* (AGG, AGA, AUA, CUA, CCC, GGA). A derivative of Tuner™ (*lacY1*) enabling low levels expression in all cells for solubility and activity enhancement.
	0.4 ml	70954-3		
	1.0 ml	70954-4		
Rosetta(DE3) Singles	11 rxn	71099-3		
	22 rxn	71099-4		
Rosetta(DE3)pLysS	0.2 ml		70951-3	
	0.4 ml	70956-3		
	1.0 ml	70956-4		
Rosetta(DE3)pLysS Singles	11 rxn	71100-3		
	22 rxn	71100-4		
Rosetta-gami™ (DE3)	0.2 ml		71062-3	Enhances expression of proteins that contain codons rarely used in *E. coli* (AGG, AGA, AUA, CUA, CCC, GGA). Allows disulfide bond formation in the *E. coli* cytoplasm.
	0.4 ml	71055-3		
	1.0 ml	71055-4		
Rosetta-gami(DE3)pLysS	0.2 ml		71064-3	
	0.4 ml	71057-3		
	1.0 ml	71057-4		
RosettaBlue™(DE3)	0.2 ml		71066-3	Enhances expression of proteins that contain codons rarely used in *E. coli* (AGG, AGA, AUA, CUA, CCC, GGA) and is a high stringency host with *recA*, *endA* and *lacI*^{q} mutations.
	0.4 ml	71059-3		
	1.0 ml	71059-4		
RosettaBlue(DE3)pLysS	0.2 ml		71068-3	
	0.4 ml	71034-3		
	1.0 ml	71034-4		
Tuner™(DE3)	0.2 ml		70612-3	Enables adjustable levels of expression in all cells due to the *lacY1* mutation; lower IPTG levels may enhance solubility and activity of the target protein.
	0.4 ml	70623-3		
	1.0 ml	70623-4		
Tuner(DE3)pLysS	0.2 ml		70613-3	
	0.4 ml	70624-3		
	1.0 ml	70624-4		

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Isogenic Host Strains for Initial Cloning, Controls, and Expression

These host strains are isogenic with the λDE3 lysogens used for protein expression but lack a source of T7 RNA polymerase. These strains can be used for T7-based expression by infection with λCE6, or for expression from *E. coli* promoters. Only the NovaBlue strain is recommended for initial cloning.

Strain	Size	Competent Cells Cat. No.	Glycerol Stock Cat. No.	Application
AD494	0.2 ml		69032-3	Allows disulfide bond formation in *E. coli* cytoplsm.
	0.4 ml	69033-3		
	1 ml	69033-4		
BL21	0.2 ml		69386-3	Protects target protein from *lon* and *ompT* proteases.
	0.4 ml	69449-3		
	1 ml	69449-4		
B834	0.2 ml		69287-3	Selenomethionine and ^{35}-S-met labeling
BLR	0.2 ml		69207-3	A *recA*$^-$ derivative of BL21 that may stabilize target plasmids containing repetitive sequences.
	0.4 ml	69052-3		
	1 ml	69052-4		
HMS174	0.2 ml		69385-3	A *recA*$^-$ K-12 strain that may stailize traget plasmids containing repetitive sequences.
	0.4 ml	69452-3		
	1 ml	69452-4		
NovaBlue	0.2 ml		69009-3	High stringency host with the r*ecA*$^-$ *endA*- and *lacI*^{q} mutations.
	0.4 ml	9825-3		
	1 ml	69825-4		
Origami™	0.2 ml		70616-3	Allows disulfide bond formation in *E. coli* cytoplsm.
	0.4 ml	70626-3		
	1 ml	70626-4		
Origami B	0.2 ml		70829-3	Allows disulfide bond formation in *E. coli* cytoplasm and protects target proteins from *lon* and *ompT* proteases. A derivative of Tuner™ (*lacY1*) enabling low levels expression in all cells for solubility and activity enhancement.
	0.4 ml	70836-3		
	1 ml	70836-4		
Rosetta™	0.2 ml		70949-3	Enhances expression of proteins that contain codons rarely used in *E. coli* (AGG, AGA, AUA, CUA, CCC, GGA). A derivative of Tuner™ (*lacY1*) enabling low levels expression in all cells for solubility and activity enhancement.
	0.4ml	70953-3		
	1 ml	70953-4		
RosettaBlue™	0.2 ml		71065-3	Enhances expression of proteins that contain codons rarely used in *E. coli* (AGG, AGA, AUA, CUA, CCC, GGA) and is a high stringency host with *recA*, *endA* and *lacI*^{q} mutations.
	0.4 ml	71058-3		
	1 ml	71058-4		
Rosetta-gami™	0.2 ml		71061-3	Enhances expression of proteins that contain codons rarely used in *E. coli* (AGG, AGA, AUA, CUA, CCC, GGA). Allows disulfide bond formation in the *E. coli* cytoplasm.
	0.4 ml	71054-3		
	1 ml	71054-4		
Tuner™	0.2 ml		70611-3	Enables adjustable levels of expression in all cells due to the *lacY1* mutation; lower levels may enhance solubility and activity of the target protein.
	0.4ml	70622-3		
	1 ml	70622-4		

pET Host Strain Competent Cell Sets

The competent cell sets are for optimizing vector/host strain combinations.

Competent Cell Set	Size	Cat. No.	Strains
(DE3) Competent Cell Set	0.2 ml each	71032-3	AD494(DE3); BL21(DE3); BL21(DE3)*trxB*; BLR(DE3); HMS174-DE3); Origami™(DE3); Origami B(DE3); NovaBlue(DE3); Rosetta™(DE3); Tuner™(DE3)
(DE3)pLysS Competent Cell Set	0.2 ml each	71033-3	AD494(DE3)pLysS; BL21(DE3)pLysS; BL21(DE3)*trxB*pLysS; BLR(DE3)pLysS; HMS174(DE3)pLysS; Origami(DE3)pLysS; Origami B(DE3)pLysS; Rosetta(DE3)pLysS; Tuner(DE3)pLysS
AD494 Competent Cell Set	0.2 ml each	70231-3	AD494; AD494(DE3); AD494(DE3)pLysS
BL21 Competent Cell Set	0.2 ml each	70232-3	BL21; BL21(DE3); BL21(DE3)plysS
BLR Competent Cell Set	0.2 ml each	70233-3	BLR; BLR(DE3); BLR(DE3)pLysS
HMS174 Competent Cell Set	0.2 ml each	70234-3	HMS174; HMS174(DE3); HMS174(DE3)pLysS
Origami Competent Cell Set	0.2 ml each	70670-3	Origami; Origami(DE3); Origami(DE3)pLysS
Origami B Competent Cell Set	0.2 ml each	70911-3	Origami B; Orgami B;(DE3); Orgami B(DE3)pLysS
Rosetta Competent Cell Set	0.2 ml each	70987-3	Rosetta; Rosetta(DE3); Rosetta(DE3)pLysS
RosettaBlue™ Competent Cell Set	0.2 ml each	71079-3	RosettaBlue; RosettaBlue(DE3); RosettaBlue(DE3)pLysS
Rosetta-gami™ Competent Cell Set	0.2 ml each	71080-3	Rosetta-gami; Rosetta-gami(DE3); Rosetta-gami(DE3)pLysS
Tuner™ Competent Cell Set	0.2 ml each	70726-3	Tuner; Tuner(DE3); Tuner(DE3)pLysS

pET System Lambda Phages

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

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