pET System Vectors and Hosts

Instruction Manual

Catalog #211521, #211523, #211621, #211623

Revision C0

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211521-12

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pET System Vectors and Hosts

CONTENTS

pET System* Vectors and Hosts

MATERIALS PROVIDED

Kit	Quantity	Catalog #	Storage
pET expression systems			
pET 3 vector series: 3a, b, c and d DNA[a,b]	Four 20-µg tubes of cesium chloride-banded, supercoiled plasmid DNA	#211621	–20°C
BL21-Gold(DE3) competent cells	10 × 100 µl		–80°C
BL21-Gold(DE3)pLysS competent cells	10 × 100 µl		–80°C
pUC18 control plasmid (0.1 ng/µl in TE buffer)	2 ×10 µl		–20°C
pET 11 vector series: 11a, b, c and d DNA[a,b,c]	Four 20-µg tubes of cesium chloride-banded, supercoiled plasmid DNA	#211623	–20°C
BL21-Gold(DE3) competent cells	10 × 100 µl		–80°C
BL21-Gold(DE3)pLysS competent cells	10 × 100 µl		–80°C
pUC18 control plasmid (0.1 ng/µl in TE buffer)	2 ×10 µl		–20°C
pET vectors			
pET 3 vector series: 3a, b, c and d DNA[a,b]	Four 20-µg tubes containing cesium chloride-banded, supercoiled plasmid DNA	#211521	–20°C
pET 11 vector series: 11a, b, c and d DNA[a,b,c]	Four 20-µg tubes containing cesium chloride-banded, supercoiled plasmid DNA	#211523	–20°C

[a] The pET 3a, b, c and pET 11a, b, c plasmids have one base pair shift in the *Bam*H I site, from a to b and b to c.

[b] The pET 3d and 11d plasmids have an *Nco* I cloning site, which is not present in a, b and c.

[c] The pET 11 series includes the *lac* operator and *lacI* repressor gene for tighter expression control (not in the pET 3 series).

STORAGE CONDITIONS

Vectors: –20°C
Competent Cells: –80°C

* The pET system is covered by U.S. Patent No. 4,952,496. A nondistribution agreement accompanies the products. Commercial customers must obtain a license agreement from Associated Universities before purchase.

ADDITIONAL MATERIALS REQUIRED

IPTG
β-Mercaptoethanol and Falcon 2059 polypropylene tubes
calf intestinal alkaline phosphatase
T4 ligase

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INTRODUCTION

The pET expression system[1] is one of the most widely used systems for the cloning and *in vivo* expression of recombinant proteins in *E. coli*. This is due to the high selectivity of the pET system's bacteriophage T7 RNA polymerase for its cognate promoter sequences, the high level of activity of the polymerase and the high translation efficiency mediated by the T7 gene *10* translation initiation signals. In the pET system, the protein coding sequence of interest is cloned downstream of the T7 promoter and gene *10* leader sequences, and then transformed into *E. coli* strains. Protein expression is achieved either by IPTG induction of a chromosomally integrated cassette in which the T7 RNA polymerase is expressed from the *lacUV5* promoter, or by infection with the polymerase-expressing bacteriophage lambda CE6.[2] Due to the specificity of the T7 promoter, basal expression of cloned target genes is extremely low in strains lacking a source of T7 RNA polymerase. Upon induction the highly active polymerase essentially out-competes transcription by the host RNA polymerase. This phenomenon, together with high-efficiency translation, achieves expression levels in which the target protein may constitute the majority of the cellular protein—after only a few hours.

pET Expression Vectors

The pET expression vectors, derived from the pBR322 plasmid, are engineered to take advantage of the features of the T7 bacteriophage gene *10* that promote high-level transcription and translation. The bacteriophage-encoded RNA polymerase is highly specific for the T7 promoter sequences, which are rarely encountered in genomes other than T7 phage genome. First, this ensures that the T7 promoter will not be recognized by host cell RNA polymerase. Thus target genes are transcriptionally silent in the uninduced state--a feature that is very important if the gene to be expressed is toxic to the cell. Second, upon induction, the target gene is the only gene in the cell that will be transcribed by the highly active polymerase.

In addition to the T7 promoter, all the vectors contain the gene *10* 5′ leader, which facilitates highly efficient translation. The protein coding sequence of interest may be cloned directly after the gene *10* initiation codon using the *Nde* I (pET-3,-11, a, b and c) or *Nco* I sites (pET-3d, and -11d). Alternatively, the pET-3 and pET-11 vectors contain *Bam*H I cloning sites in all three reading frames relative to the gene *10* reading frame. Cloning the gene of interest using the *BamH* I site results in a fusion protein containing 13 N-terminal amino acids from gene *10*. The gene *10* transcription terminator is also included downstream of the cloning sites to allow efficient termination of transcription, preventing transcriptional read-through of unwanted plasmid sequences and increasing the RNA polymerase density on the sequence of interest--allowing high level accumulation of the specific protein-coding RNA transcripts.

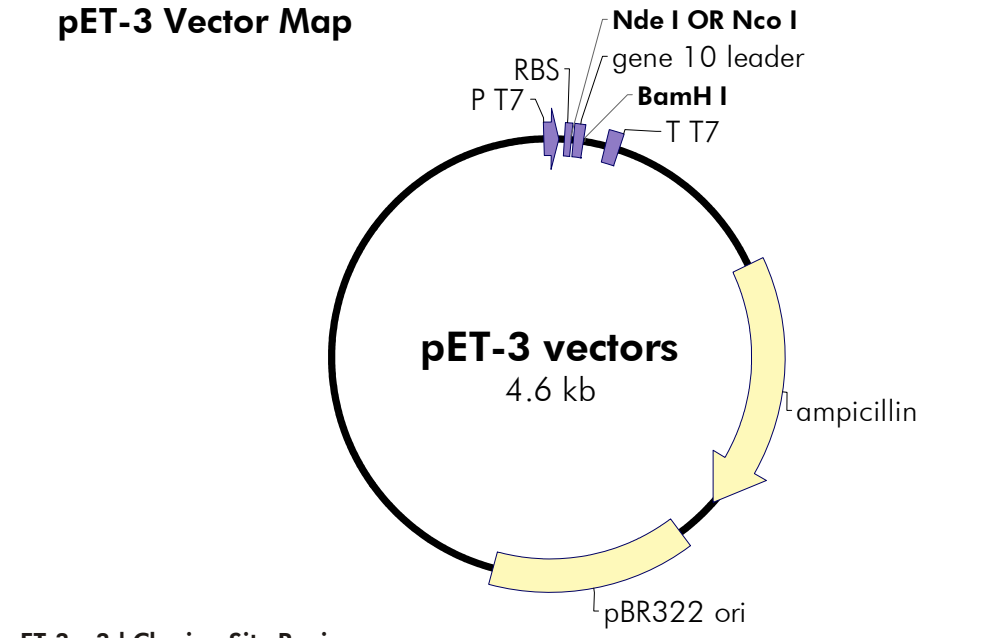
Despite the strong selectivity of the T7 promoter for its phage-encoded polymerase, residual "leaky" expression of very toxic proteins from the basic pET-3 constructs can be lethal to the cell. To circumvent this problem and achieve more stringent control of expression, the *lac* operator has been inserted between the T7 promoter and translation initiation sequences in the pET-11 vectors, thereby allowing IPTG-mediated de-repression of the T7 promoter in addition to IPTG-induction of T7 polymerase from the *lacUV5* promoter in the DE3 containing strains (see *Bacterial Strains*). In order to provide adequate levels of *lacI* protein to shut off T7 polymerase expression as well as T7 promoter transcription, the *lacI* gene is included on the pET-11 plasmids.

All of the vectors in the pET-3 and pET-11 series contain the β-lactamase gene for ampicillin resistance and the pBR322 origin of replication. Other features specific to the various vectors are listed in Table I below and in Figures 1 and 2.

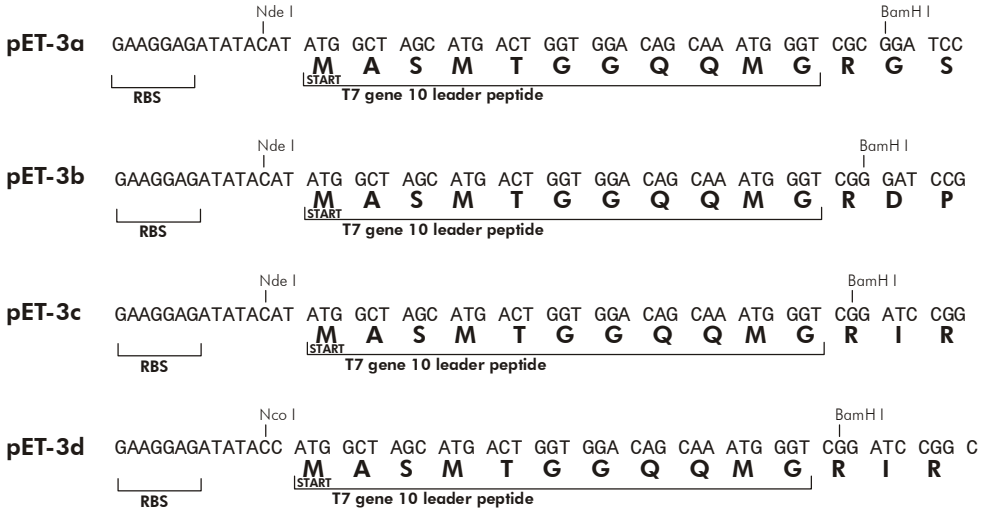
TABLE I
Features of the pET System Vectors

Vector	Promoter	ATG cloning site	Reading frame
pET-3a	T7	*Nde* I	GGA[a]
pET-3b	T7	*Nde* I	GAT
pET-3c	T7	*Nde* I	ATC
pET-3d	T7	*Nco* I	ATC
pET-11a	T7/lac O	*Nde* I	GGA
pET-11b	T7/lac O	*Nde* I	GAT
pET-11c	T7/lac O	*Nde* I	ATC
pET-11d	T7/lac O	*Nco* I	ATC

[a] Reading frame defined as the first full codon within the *Bam*H I recognition sequence (GGATCC) that is in the same frame as the gene *10* initiation codon.



pET-3a–3d Cloning Site Regions
sequence shown (66–122)

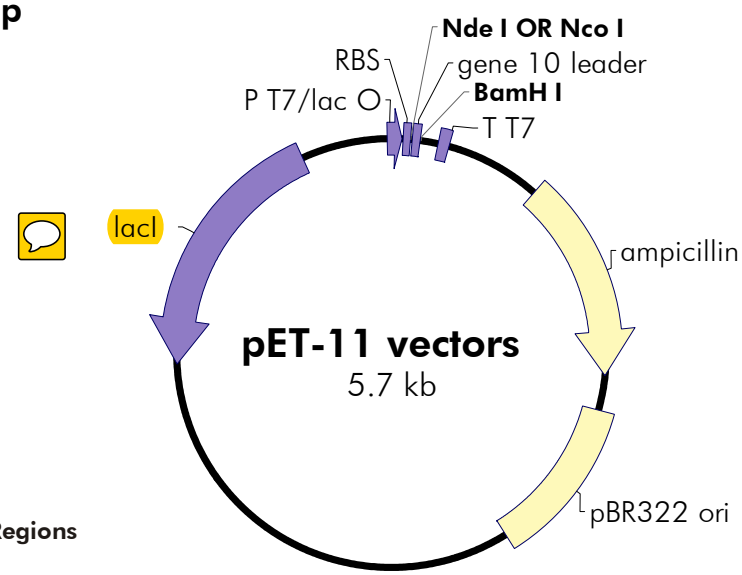


Feature	Nucleotide Position			
	pET-3a	**pET-3b**	**pET-3c**	**pET-3d**
T7 promoter	1–19	1–19	1–19	1–19
ribosome binding site (RBS)	66–72	66–72	66–72	66–72
Nde I (pET-3a–c) or Nco I (pET-3d) cloning site	78–83	78–83	78–83	78–83
T7 gene 10 translated leader	81–113	81–113	81–113	80–112
BamH I cloning site	117–122	116–121	115–120	114–119
T7 terminator	191–237	190–236	189–235	188–234
ampicillin resistance (*bla*) ORF	840–1697	839–1696	838–1695	837–1694
pBR322 origin of replication	1848–2515	1847–2514	1846–2513	1845–2512

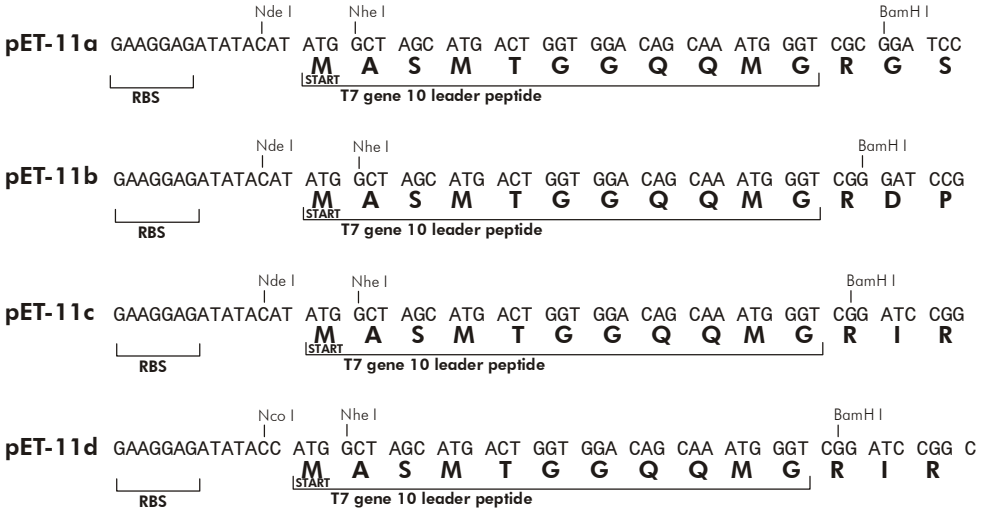
FIGURE 1 The pET-3 vectors

BPI submitted vector sequence change notice indicating 3 bp deletion at position 2512-2514 and 1 bp substitution at 3678 of currently published sequence and that lacI in vector is wt and not lacIq.

pET-11 Vector Map



pET-11a–11d Cloning Site Regions
sequence shown (74–130)



Feature	Nucleotide Position			
	pET-11a	pET-11b	pET-11c	pET-11d
T7 promoter with *lac* operator	1–43	1–43	1–43	1–43
ribosome binding site (RBS)	74–80	74–80	74–80	74–80
Nde I (pET-11a–c) or Nco I (pET-11d) cloning site	86–91	86–91	86–91	86–91
T7 gene 10 translated leader	89–121	89–121	89–121	88–120
BamH I cloning site	125–130	124–129	123–128	122–127
T7 terminator	199–245	198–244	197–243	196–242
ampicillin resistance (*bla*) ORF	657–1514	656–1513	655–1512	654–1511
pBR322 origin of replication	1665–2332	1664–2331	1663–2330	1662–2329
lacI repressor ORF	4209–5288	4208–5287	4207–5286	4206–5285

FIGURE 2 The pET-11 vectors

Position of lacI changed by 3 bp in all variants. This is only element downstream of 3 bp deletion.

Bacterial Strains

The BL21-Gold expression strains provided with catalog #211621 and #211623 are derivatives of the *E. coli* B strain BL21 (F^- *ompT* r^-_B m^-_B). The BL21 strain is generally good for protein expression due to its deficiency in *lon* protease[3] as well as the *ompT* outer membrane protease that can degrade proteins during purification. (For more information, see Table II *Features of the BL21-Derived Expression Strains*.) BL21 strains are rifampicin sensitive, allowing use of the drug to inhibit transcription of host cell polymerase in instances where background synthesis is undesirable.

The strain BL21-Gold (DE3) [F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte] carries a lambda DE3 lysogen that has the phage 21 immunity region, the *lacI* gene and the *lacUV5*-driven T7 RNA polymerase expression cassette. Upon induction with IPTG, the *lacUV5* promoter is de-repressed allowing over-expression of T7 RNA polymerase and thus expression of the T7-promoted target gene from the pET expression plasmid.

In order to further reduce basal activity of T7 RNA polymerase in the uninduced state, the strain BL21-Gold(DE3) [F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte (pLysS Cam^r)] carries a derivative of the plasmid pACYC184, which expresses the T7 lysozyme gene at low levels. T7 lysozyme binds to T7 RNA polymerase and inhibits transcription by this enzyme. Upon IPTG induction, overproduction of the T7 RNA polymerase renders low level inhibition by T7 lysozyme virtually ineffective. In addition to inactivation of T7 RNA polymerase transcription, T7 lysozyme has a second function involving specific cleavage of the peptidoglycan layer of the *E. coli* outer wall. The inability of T7 lysozyme to pass through the bacterial inner membrane restricts the protein to the cytoplasm, allowing *E. coli* to tolerate expression of the lysozyme protein. This second function of lysozyme does, however, confer the advantage of allowing cell lysis under mild conditions. Cells expressing T7 lysozyme are subject to lysis under conditions that would normally only disrupt the inner membrane (e.g., freeze–thaw cycles or the addition of chloroform or a mild detergent such as 0.1% Triton X-100) due to the action of the protein on the outer wall when the inner membrane is disrupted.

The BL21-Gold expression strains incorporate major improvements over the original BL21 strain. The BL21-Gold strains feature the Hte phenotype present in our highest efficiency strain, XL10-Gold ultracompetent cells.[4] The presence of the Hte phenotype increases the transformation efficiency of the BL21-Gold cells to >1 × 10^8 cfu/µg of pUC18 DNA. In addition, the gene that encodes endonuclease I (*endA*), which rapidly degrades plasmid DNA isolated by most miniprep procedures, is inactivated. These two improvements allow direct cloning of many protein expression constructs.

In cases where the protein of interest is extremely toxic to *E. coli* or where clones obtained from direct transformation of BL21-Gold strains consistently show sequence anomalies, perform the initial cloning steps in cells in which the T7 promoter is inactive and that are *RecA*$^-$(e.g. XL1-Blue competent cells). Strains carrying the *lacI*^{q} gene may be useful for further inhibiting basal expression from the pET-11 vectors during subcloning.

TABLE II

Features of the BL21-Derived Expression Strains[5]

Expression Strain	Induction	Advantages	Disadvantages
BL21-Gold (DE3) competent cells	IPTG induction of T7 polymerase from *lacUV5* promoter	High-level expression; Ease of induction, direct cloning in expression strain	Leaky expression of T7 polymerase can lead to uninduced expression of potentially toxic proteins.
BL21-Gold (DE3)pLysS competent cells	IPTG induction of T7	Ease of induction, reduced uninduced expression, direct cloning in expression strain	Slight inhibition of induced expression when compared with BL21-Gold (DE3).
BL21 competent cells	Infection with lambda bacteriophage CE6	Tightest control of uninduced expression	Induction not as efficient as DE3 derivatives. Induction (infection) process is more cumbersome.

Bacteriophage CE6

In cases where target genes are too toxic to allow plasmids to be established in DE3 lysogens, T7 RNA polymerase can be delivered to the cell by infection with the bacteriophage CE6. By this method, no T7 RNA polymerase is present in the cell until the desired time of induction. CE6 expresses T7 RNA polymerase from the lambda pL and pI promoters, and carries the *Sam7* lysis mutations. This bacteriophage will allow effective expression of target genes in BL21 cells, and presumably other nonrestricting hosts which absorb lambda. The phage can be propagated in the host strain LE392 [F$^-$e14$^-$ (mcrA) hsdR514 ($r_K^-m_K^+$) supE44 supF58],[6] which suppresses the *Sam7* mutation and therefore allows lysis of infected cells.

CLONING PROTOCOL

Preparing the Vectors

- Perform a complete DNA digestion with the appropriate enzymes. (Perform *Nde* I–*Bam*H I or *Nco* I–*Bam*H I double digests to clone the gene of interest at the initiation codon. Perform a *Bam*H I single digest to produce a fusion of the protein of interest to the gene 10 translated leader.) If the inserts to be cloned into these vectors contain one or more internal *Nde* I, *Nco* I or *BamH* I sites, PCR primers may be engineered to include restriction sites with overhangs compatible with the overhangs produced by the chosen enzymes.

- Dephosphorylate the digested pET vector with calf intestinal alkaline phosphatase (CIAP) prior to ligating to the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the digested vector DNA on an agarose gel and gel purifying the desired vector band, leaving behind the small fragment excised from between the two restriction enzyme sites.

- After gel purification, resuspend the vector DNA in a volume of TE buffer (see *Preparation of Media and Reagents*) that will allow the concentration of the vector DNA to be the same as the concentration of the insert DNA (~0.1μg/μl).

Ligating the Insert

For ligation, the ideal insert-to-vector ratio of DNA is variable; however, a reasonable starting point is 2:1 (insert-to-vector molar ratio), measured in available picomole ends. This is calculated as follows:

$$\text{Picomole ends/microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 600}$$

1. Prepare three control and two experimental 10-µl ligation reactions by adding the following components to separate sterile 1.5-ml microcentrifuge tubes:

   Note *For blunt-end ligation, reduce the rATP to 0.5 mM and incubate the reactions overnight at 12–14°C.*

	Control				Experimental	
Ligation reaction components	**1**[a]	**2**[b]	**3**[c]		**4**[d]	**5**[d]
Prepared vector (0.1 µg/µl)	1.0 µl	1.0 µl	0.0 µl		1.0 µl	1.0 µl
Prepared insert (0.1 µg/µl)	0.0 µl	0.0 µl	1.0 µl		*X* µl	*X* µl
rATP [10 mM (pH 7.0)]	1.0 µl	1.0 µl	1.0 µl		1.0 µl	1.0 µl
Ligase buffer (10×)[e]	1.0 µl	1.0 µl	1.0 µl		1.0 µl	1.0 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.0 µl	0.5 µl		0.5 µl	0.5 µl
Double-distilled (ddH_2O) to 10 µl	6.5 µl	7.0 µl	6.5 µl		*X* µl	*X* µl

[a] This control tests for the effectiveness of the digestion and the CIAP treatment. Expect a low number of transformant colonies if the digestion and CIAP treatment are effective.

[b] This control indicates whether the vector is cleaved completely or whether residual uncut vector remains. Expect an absence of transformant colonies if the digestion is complete.

[c] This control verifies that the insert is not contaminated with the original vector. Expect an absence of transformant colonies if the insert is pure.

[d] These experimental ligation reactions vary the insert-to-vector ratio. Expect a majority of the transformant colonies to represent recombinants.

[e] See *Preparation of Media and Reagents*.

2. Incubate the reactions for 2 hours at room temperature (22°C) or overnight at 4°C.

3. Transform 1–2 µl of the ligation mix into the appropriate competent bacteria. Plate on selective media.

   Note *For most applications, the ligation reaction may be transformed directly into BL21-Gold(DE3) or BL21-Gold(DE3)pLysS competent cells (provided with catalog #211621 and #211623). See* Transformation of the BL21-Gold Expression Strains *for a transformation protocol.*

4. Verify the presence and orientation of the insert by PCR, restriction analysis or other appropriate methods.

TRANSFORMATION PROTOCOL

Transformation of the BL21-Gold Expression Strains

1. Thaw the competent cells on ice.

   Note *Store the competent cells **on ice at all times** while aliquoting. It is essential that the Falcon 2059 polypropylene tubes are placed on ice before the competent cells are thawed and that 100 µl of competent cells are aliquoted directly into each **prechilled** polypropylene tube. Do not pass the frozen competent cells through more than one freeze–thaw cycle.*

2. Gently mix the competent cells. Aliquot 100 µl of the competent cells into the appropriate number of prechilled 15-ml Falcon 2059 polypropylene tubes.

3. Add 1–50 ng of DNA to each transformation reaction and swirl gently. For the control transformation reaction, add 1 µl of the pUC18 control plasmid (provided with Catalog #211621 and #211623) to a separate 100-µl aliquot of the competent cells and swirl gently.

4. Incubate the reactions on ice for 30 minutes.

5. Preheat SOC medium[§] in a 42°C water bath for use in step 8.

6. Heat-pulse each transformation reaction in a 42°C water bath for 20 seconds. **The duration of the heat pulse is critical for optimal transformation efficiencies.**

7. Incubate the reactions on ice for 2 minutes.

8. Add 0.9 ml of 42°C SOC medium to each transformation reaction and incubate the reactions at 37°C for 1 hour with shaking at 225–250 rpm.

9. Concentrate the cells from the experimental transformation by centrifugation (200 × *g* for 3–5 minutes) and plate the entire transformation reaction (using a sterile spreader)[‖] onto a single LB–ampicillin agar plate.[§]

   To plate the cells transformed with the pUC18 control plasmid, first place a 195-µl pool of SOC medium on an LB–ampicillin agar plate.[§] Add 5 µl of the control transformation reaction to the pool of SOC medium. Use a sterile spreader to spread the mixture.

10. Incubate the plates overnight at 37°C.

[§] See *Preparation of Media and Reagents*.

[‖] When spreading bacteria onto the plate, tilt and tap the spreader to remove the last drop of cells. If plating <100 µl of the transformation reaction, plate the cells in a 200-µl pool of SOC medium. If plating ≥100 µl, the cells can be spread directly onto the plates.

Transformation Summary for the pUC18 Control Plasmid

Host strain	Plating quantity	Expected number of colonies	Efficiency (cfu/μg of pUC18 DNA)
BL21-Gold(DE3) competent cells	5 μl	>50	≥1 × 10^8
BL21-Gold(DE3)pLysS competent cells	5 μl	>50	≥1 × 10^8

[a] The efficiencies quoted for the BL21-Gold(DE3) and the BL21-Gold(DE3)pLysS host strain competent cells (provided with Catalog #211621 and #211623) are guaranteed only if the user follows the storage and transformation protocols outlined in this manual. Efficiencies of competent cells prepared by the user cannot be guaranteed.

EXPRESSION PROTOCOLS

Induction of Target Protein Using IPTG

The following induction protocol is a general guide for expression of genes under the control of IPTG-inducible promoters on an analytical scale (1 ml of induced culture). Most commonly, this protocol is used to analyze protein expression of individual transformants when using BL21-Gold(DE3) host strains in combination with plasmids containing T7 promoter constructs (e.g. pET vectors). Expression cassettes under the control of the *trp*/*lac* hybrid promoter, *tac*, can be also induced using this protocol. In the case of *tac* promoter constructs, non-DE3 lysogen strains can be employed as hosts.

Note *The transformation procedure described above will produce varying numbers of colonies depending on the efficiency of transformation obtained using the expression plasmid. It is prudent to test more than one colony as colony-to-colony variations in protein expression are possible.*

1. Inoculate 1-ml aliquots of LB broth[§] containing 100 μg/ml of carbenicillin or ampicillin with single colonies from the transformation. Shake at 220–250 rpm at 37°C overnight.

   Note *If the transformed cells contain a pACYC-based plasmid (e.g., the BL21-Gold(DE3)pLysS strain or any BL21-CodonPlus strain), the overnight culture must include chloramphenicol at a final concentration of 50 μg/ml in addition to the carbenicillin/ampicillin required to maintain the pET plasmid.*

2. The next morning, pipet 50 μl of each culture into fresh 1-ml aliquots of LB broth containing no selection antibiotics. Incubate these cultures with shaking at 220–250 rpm at 37°C for 2 hours.

[§] See *Preparation of Media and Reagents.*

3. Pipet 100 µl of each of the cultures into clean microcentrifuge tubes and place the tubes on ice until needed for gel analysis. These will serve as the non-induced control samples.

4. To the rest of the culture in each tube add IPTG to a final concentration of 1 mM. Incubate with shaking at 220–250 rpm at 37°C for 2 hours.

   Note *These values for IPTG concentration and induction time are starting values only and may require optimization depending on the gene expressed.*

5. After the end of the induction period, place the cultures on ice.

6. Pipet 20 µl of each of the induced cultures into clean microcentrifuge tubes. Add 20 µl of 2× SDS gel sample buffer[§] to each.

7. Mix the tubes containing the non-induced samples to resuspend the cells and pipet 20 µl from each tube into clean microcentrifuge tubes. Add 20 µl of 2× SDS gel sample buffer to each.

8. Heat all tubes to 95°C for 5 minutes and analyze the samples by Coomassie Brilliant Blue staining of an SDS-PAGE gel, placing associated non-induced/induced samples in adjacent lanes.

Induction of Target Protein by Infection with Lambda CE6

Expression of genes under the control of the T7 promoter (e.g. genes in pET vectors) can be achieved in non-DE3 lysogen host strains (e.g. BL21) if the strain harboring the expression plasmid is subsequently infected with lambda CE6. Lambda CE6 expresses T7 polymerase, which in turn drives the transcription of the gene downstream of the T7 promoter. The following protocols describe the growth and maintenance of lambda CE6 and the use of lambda CE6 for infecting host strains. We offer the Lambda CE6 Induction Kit (Catalog# 235200) for use in protein expression protocols which incorporate CE6 infection.

[§] See Preparation of Media and Reagents.

Growth and Maintenance of High-Titer Bacteriophage Lambda CE6 Stocks

1. Inoculate 5 ml of modified* NZY broth§ with a single colony of LE392 host cells. Shake overnight at 37°C at 220–250 rpm.
2. Centrifuge the overnight culture for 15 minutes at 1700–2000 × g at 4°C. Resuspend the cells in 10 mM $MgSO_4$ to a final OD_{600} of 0.5.
3. Combine 250 µl of cells (at OD_{600} = 0.5) with 1 × 10^6 pfu of CE6 in Falcon 2059 polypropylene tubes in triplicate. Incubate at 37°C for 15 minutes.
4. Add 3 ml of melted NZY top agar§ (equilibrated to 48°C) to each cell suspension and plate on warm agarose plates.§ Incubate the plates overnight at 37°C.
5. Flood each plate with 5 ml of SM solution§ and rock the plates for 2 hours at room temperature.
6. Remove the SM solution (which contains the lambda CE6) from each plate and pool the volumes in a 50-ml conical tube.
7. Centrifuge the SM solution at 1700–2000 × *g* for 15 minutes at 4°C.
8. Remove the supernatant and determine the titer of the solution.
9. Store the lambda CE6 stock at 4°C.

Phage Amplification

If the titer drops over time, or if more phage are needed, grow up LE392 cells in 10 ml of medium and add bacteriophage lambda CE6 at a multiplicity of infection of 1:1000 (CE6-to-cell ratio). Continue growing the culture at 37°C for 5–6 hours and spin down the cellular debris. Titer of the supernatant should be ≥5.0 × 10^9 pfu/ml. For general information regarding phage amplification, see reference 7.

Induction of Target Protein by Infection with Lambda CE6

Note *This protocol is designed for inductions in 50-ml culture volumes. If inductions of larger volumes of culture are desired, it will be necessary to increase the volume of the overnight culture in step 1. The increased volume of overnight culture is necessary to achieve the required cell density (A_{600} ≤1) in the larger volume of broth the following day.*

* NZY broth to be used for lambda infection protocols should be supplemented with maltose at a final concentration of 0.2%. Add 1 ml of 20% maltose solution (filter-sterilized) per 100 ml of NZY broth to achieve the correct final concentration of maltose.

§ See *Preparation of Media and Reagents.*

1. Inoculate 5 ml of modified* NZY broth containing 100 μg/ml of carbenicillin or ampicillin with a single colony of BL21 cells (not a DE3 lysogen) harboring the expression plasmid. Shake overnight at 37°C at 200–250 rpm.

   Note *If the host cells contain a pACYC-based plasmid (e.g., any BL21-CodonPlus strain), the overnight culture must include chloramphenicol at a final concentration of 50μg/ml in addition to the carbenicillin/ampicillin required to maintain the pET plasmid.*

2. In the morning, centrifuge 1.0 ml of the overnight culture, resuspend the cells in 1.0 ml of fresh modified* NZY broth, and pipet the resuspended cells into a flask containing 50 ml of fresh modified* NZY broth (no selection antibiotics).

3. Record the A_{600} of the diluted culture. It should be ≤ 0.1. If the A_{600} is > 0.1, use more fresh modified* NZY broth to dilute the culture to A_{600} ≤ 0.1. If the A_{600} is < 0.1, the time required to reach an A_{600} of 0.3 (in step 4) will be extended.

4. Grow the culture to an A_{600} of 0.3 and add glucose to a final concentration of 4 mg/ml (e.g. 1.0 ml of a 20% glucose solution to the 50-ml culture).

5. Grow the culture to an A_{600} of 0.6–1.0 and add $MgSO_4$ to a final concentration of 10 mM (e.g. 500 μl of a 1.0 M solution of $MgSO_4$ to the 50-ml culture).

6. Remove a portion of the culture to serve as the uninduced control and infect the rest with bacteriophage lambda CE6 at a multiplicity of infection (MOI) of 5–10 particles per cell. (To optimize induction, cultures may be split into 3 or 4 aliquots and infected with varying dilutions of bacteriophage lambda CE6. The subsequent induction can be monitored by SDS-PAGE or by a functional assay, if available.)

7. Grow the culture for 2–3 hours.

8. Remove 5–20 μl of the culture for determination by SDS-PAGE, and harvest the remaining culture by centrifugation. Store the pellets at –70°C.

   Note *If induction will be monitored using Coomassie stain, silver stain, or another nonspecific protein stain, include a control of CE6-infected BL21 cells harboring the plasmid without a cloned insert.*

* NZY broth to be used for lambda infection protocols should be supplemented with maltose at a final concentration of 0.2%. Add 1 ml of 20% maltose solution (filter-sterilized) per 100 ml of NZY broth to achieve the correct final concentration of maltose.

TROUBLESHOOTING

Observation	Suggestions
Poor induction	Poor induction can occur for a number of reasons, including loss of plasmid due to instability resulting from expression of toxic proteins, unstable mRNA or poorly translated RNA that is high in secondary structure, and high abundance of codons which are rare in *E. coli*. BL21-CodonPlus Competent Cells are engineered to contain extra copies of genes that encode the tRNAs that most frequently limit translation of heterologous proteins in *E. coli*.
Plasmid instability	Prior to induction of cultures, determine the fraction of cells containing inducible plasmid by plating a 10^5–fold dilution of cells on plates that have a) IPTG plus ampicillin, b) IPTG alone, and cells at a dilution of 2×10^6–fold which have c) ampicillin or d) no additives Reduced colony formation should occur on ampicillin-containing plates relative to the LB plates. Successful induction of target gene on IPTG-containing plates should result in virtually no increase in bacterial growth; therefore, <2% of the cells should form colonies on plates that contain IPTG alone and 0.01% on plates that contain IPTG plus antibiotic. Conversely, cells that contain mutant plasmids that are poorly induced should show higher colony formation on the IPTG plates.
	More tightly controlled induction may be desirable. Available methods include using a pET-11 vector (instead of a pET-3 vector) containing a *lac* operator; using BL21-Gold(DE3)pLysS (instead of BL21-Gold(DE3)); and, in extreme cases, performing induction by infection with the bacteriophage CE6. Also see Table II, *Features of the BL21-Derived Expression Strains*.
Problems associated with induction time	In certain cases accumulation of target protein may kill cells at saturation while allowing normal growth in logarithmically growing cultures, while in other cases target protein may continue to accumulate in cells well beyond the recommended 3-hour induction period. To determine the optimal time of induction, a time course may be carried out during which a small portion of the culture is analyzed by SDS-PAGE at various times following induction.
Inclusion bodies	In some cases protein may form insoluble inclusion bodies at 37°C. In many cases, this protein may be soluble and active if the induction is carried out at 30°C. Inclusion body formation may be used as a purification step by simply spinning out the insoluble material from crude lysates and redissolving the protein in urea or guanidinium-HCl.

PREPARATION OF MEDIA AND REAGENTS

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Adjust to pH 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Adjust pH to 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin Broth (per Liter)

1 liter of LB broth, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin–Methicillin Agar (per Liter)

(Use for reduced satellite colony formation)
1 liter of LB agar
Autoclave
Cool to 55°C
Add 20 mg of filter-sterilized ampicillin
Add 80 mg of filter-sterilized methicillin
Pour into petri dishes (~25 ml/100-mm plate)

NZY broth, (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Adjust the pH to 7.5 with NaOH

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

SOB Medium (per Liter)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Autoclave
Add 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$/liter of SOB medium prior to use
Filter sterilize

SOC Medium (per 100 ml)

SOB medium
Add 1 ml of a 2 M filter-sterilized glucose solution or 2 ml of 20% (w/v) glucose prior to use
Filter sterilize

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction.*

Agarose Plates (per Liter)

Melt 20 g of agarose in 500 ml of deionized H_2O
Add the following:
5 g of NaCl
5 g of yeast extract
10 g of tryptone
Add deionized H_2O to a final volume of 1 liter
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

NZY Top Agar (per Liter)

Prepare 1 liter of NZY broth
Add 0.7% (w/v) agarose
Autoclave

SM Solution

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
50 ml of 1 M Tris-HCl (pH 7.5)
5 ml 2% gelatin
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5
Autoclave

2× SDS gel sample buffer

100 mM Tris-HCl (pH 6.5)
4% SDS (electrophoresis grade)
0.2% bromophenol blue
20% glycerol

Note *Add dithiothreitol to a final concentration in the 2× buffer of 200 mM prior to use.* ***This sample buffer is useful for denaturing, discontinuous acrylamide gel systems only.***

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.chem.agilent.com/en-US/search/library/Pages/MSDSSearch.aspx*. MSDS documents are not included with product shipments.

pET System Vectors and Hosts

Catalog #211521, #211523, #211621, and #211623

QUICK-REFERENCE PROTOCOL

- Prepare DNA insert containing the target gene
- Prepare pET vector by digestion with the appropriate restriction enzyme(s), CIAP treatment and agarose gel purification
- Ligate the prepared vector and insert DNA
- Transform cloning host competent cells (e.g. Agilent XL1-Blue cells) and verify construct

Expression in *E. coli*

- Transform BL21 expression host competent cells
- Induce protein expression by IPTG addition or CE6 infection
- Harvest cells; analyze cell pellets for target protein production
- Purify target protein

Expression *in vitro*

- Add recombinant pET plasmid DNA or PCR-amplified DNA to coupled transcription/translation reaction
- Purify target protein