Novagen®

pETBlue™ System Manual

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A. Description

The unique pETBlue System combines the visual identification of recombinants and high plasmid copy number of popular blue/white screening vectors; with the tightly controlled high yield protein expression obtained with pET vectors. Blue/white screening is achieved using the weak constitutive *E. coli tet* promoter to drive expression of the *lacZ* α-peptide, whereas expression of target genes is driven by a T7*lac* promoter (1) in the opposite orientation. Insertion of target sequences into the multiple cloning site (MCS) disrupts expression of the *lacZ* α-peptide and produces a white colony phenotype in the NovaBlue strain when plated in the presence of X-gal and IPTG (IPTG is required to induce the ω-fragment of *lacZ* encoded on the F'). Colonies derived from the unmodified vector turn blue. IPTG induction at this stage does not lead to expression of target genes under the control of the T7*lac* promoter because NovaBlue does not carry the gene for T7 RNA polymerase. Because T7-driven expression requires inserts to be cloned in an antisense orientation relative to the *tet* promoter, basal expression of target sequences is virtually absent. The high copy number pUC origin of replication present on the pETBlue plasmids greatly increases plasmid yields relative to the pET vectors and provides an advantage for sequencing, mutagenesis, and other plasmid manipulations.

Target genes in pETBlue vectors can be expressed at high levels, provided that the inserted sequences are in the sense orientation relative to the T7*lac* promoter, and meet the translation requirements defined by each vector. Protein expression is accomplished in one of two ways: by infection of the NovaBlue host with λCE6 (a phage that expresses T7 RNA polymerase under the control of the λp_L promoter (2), or by transformation of the recombinant pETBlue plasmid into a (DE3)pLacI expression host strains followed by induction with IPTG. These hosts carry a chromosomal copy of T7 RNA polymerase under the control of the *lacUV5* promoter (2, 3) and supply sufficient *lac* repressor from the compatible pLacI plasmid to ensure stringent repression in the uninduced state.

B. Components

pETBlue Systems

- 20 µg pETBlue DNA (uncut)
- 500 pmol pETBlueUP primer
- 500 pmol pETBlueDOWN primer
- 0.2 ml NovaBlue Competent Cells
- 0.2 ml Tuner™(DE3)pLacI Competent Cells
- 2 × 2 ml SOC Medium
- 10 µl Test Plasmid, 0.2 ng/µl (Amp^R)

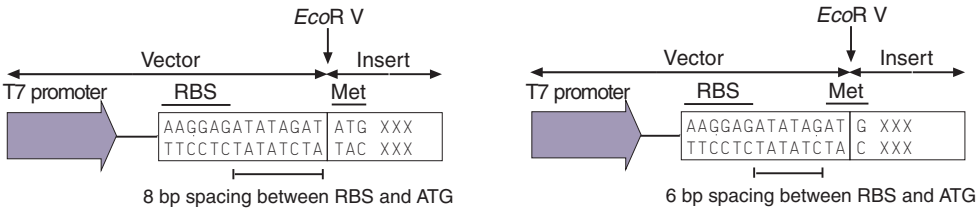
C. Storage

Store competent cells at –70°C. Store all other components at –20°C.

II. Cloning Strategies

A. pETBlue™-1

pETBlue-1 facilitates the expression of unfused, native proteins from inserts that start with an ATG initiation codon at the 5'-end and encode an open reading frame. The *Eco*R V (GATATC) blunt cloning site in this vector is optimally positioned relative to the strong T7 gene *10* ribosome binding site (RBS). Because ATG start codons must be positioned 5–11 bp downstream of the RBS to obtain efficient translation initiation, **the *Eco*R V cloning site must be used for T7 promoter-mediated expression in pETBlue-1.** As shown in the diagram below, there are two alternative designs that allow optimal expression. The first option, shown in the left panel, is for the insert to begin with an ATG (Met) start codon, which creates an 8-bp spacing between the RBS and ATG. The other option, shown in the right panel, is for the insert to begin with a G-nucleotide that completes the ATG triplet, which creates a 6-bp spacing between the RBS and ATG. Both configurations appear to produce similar expression levels.



Inserts compatible with blunt cloning into the *Eco*R V site may be generated in the following ways:

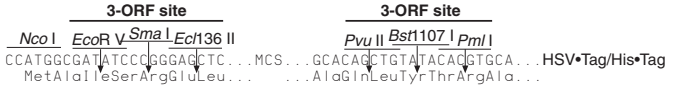
- by PCR amplification using a thermostable polymerase possessing 3'→5' exonuclease activity
- by filling in and/or polishing overhangs generated by restriction enzyme digest, random shearing, or PCR amplification with thermostable polymerases lacking 3'→5' exonuclease activity

The pETBlue-1 Perfectly Blunt® Cloning Kits, which contain pretested *Eco*R V digested, dephosphorylated pETBlue-1 vector are available for convenient cloning of these inserts.

Another cloning strategy for generating recombinants in pETBlue-1 is the use of the pETBlue-1 **A**ccep**T**or™ Vector Kit (Cat. No. 70598-3), which contains the *Eco*R V linearized vector having single 3'-dU overhangs. The **A**ccep**T**or Vector is compatible with PCR products of DNA polymerases that lack 3'→5' exonuclease activity (e.g., Nova*Taq* DNA polymerase), because many of the reaction products contain single 3'-dA overhangs. Such inserts can be directly ligated with the **A**ccep**T**or Vector without the need for prior treatment. The dU residues on the vector are converted to dT residues by the DNA repair machinery of the cell following transformation. If this strategy is used, the 5' primer should be designed according to the left panel above; i.e., the first three nucleotides should specify ATG, and the spacing between the RBS and ATG will be 9 bp.

B. pETBlue-2

pETBlue-2 provides a vector-encoded ATG start codon and wide array of downstream restriction sites, including two enzymes, *Sse*8387 I and *Not* I, specific for rare restriction sites and an *Nco* I site 8 bp downstream of the RBS that facilitates the production of native target proteins. In addition, the multiple cloning site (MCS) features dual three open reading frame (3-ORF) sites that allow ANY blunt insert to be cloned in-frame with the vector-defined ORF. Each 3-ORF site encodes overlapping restriction enzyme sites that leave blunt ends terminating in every position of the codon triplet (see diagram on the next page). Selection of the appropriate combination of sites allows an insert to be cloned in-frame with the vector-defined ORF at both its N- and C-termini. Therefore, inserts that lack a stop codon can be fused to the optional C-terminal HSV•Tag® and His•Tag® detection and purification tags if desired. The HSV•Tag is an 11 amino acid epitope derived from herpes simplex virus glycoprotein D and can be used for sensitive, specific Western blot detection with HSV•Tag® Monoclonal Antibody (Cat. No. 69171-3). The His•Tag® sequence can be used for Western detection with the His•Tag Monoclonal Antibody (Cat. No.70796-3) or for purification with His•Bind® Resins (see User Protocols TB054 and TB278).



Convenient restriction sites for reading frame adjustment at both ends of pETBlue-2 inserts
Shown are the 5´ and 3´ 3-ORF site regions of the multiple cloning sites (MCS) in pETBlue-2. The amino acid sequence of T7-driven expressed proteins is shown, including the reading frame for C-terminal HSV•Tag®/His•Tag® fusions.

Cloning strategy to generate unfused target proteins from pETBlue™-2

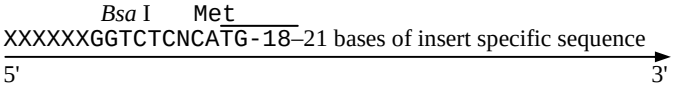
pETBlue-2 facilitates the production of unfused target proteins if desired. The vector encodes a unique *Nco* I site (CCATGG) at the N-terminal end of the MCS-defined (ORF). The ATG triplet within this site encodes the N-terminal methionine start codon in T7 RNA polymerase transcripts. Target genes or PCR-engineered inserts that contain either *Nco* I sites or sites that generate compatible overhangs [*Bsp*H I (TCATGA) and subsets of *Afl* III (ACRYGT) and *Sty* I (CCWWGG)] at the beginning of their ORF can be cloned into the pETBlue-2 *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 these cases it may be possible to employ an alternative strategy to allow the generation of native target protein. Several restriction enzymes that cleave "downstream" of their recognition site are commercially available (see table below).

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

Any of these restriction sites can be engineered into PCR primers such that *Nco* I-compatible overhangs can be generated. 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.

Example: PCR primer encoding a *Bsa* I site that generates an *Nco* I compatible overhang:



Because *Bsa* I cleaves 1 bp "downstream" of its recognition site on the top strand and 5 bp downstream on the bottom strand, it will generate an *Nco* I-compatible overhang without dictating the sequence of the 3'-flanking base, i.e., the second codon triplet is completely unrestricted. In this way, a pETBlue-2 recombinant encoding a native N-terminus of the target protein can be constructed. Creating an unfused C-terminus of the target protein is accomplished by including a stop codon in the target gene fragment.

PCR cloning strategy without restriction digestion

For convenient cloning of PCR products without the need for restriction digestion, the pETBlue™-2 vector is also available in linearized form in the Perfectly Blunt® Cloning Kits. The blunt cloning approach uses the *Eco*R V site, which is located such that the insert will specify the fourth amino acid following Met-Ala-Ile at the N-terminus of the expressed protein.

To achieve the correct reading frame in the pETBlue-2 Blunt Vector, the 5' end of the insert (and the sense PCR primer) should begin with the third base of the Ile/Met codon. To express a target protein fused with C-terminal HSV•Tag® and His•Tag® peptides, the antisense primer should begin with two bases in any combination except TA or CA, and specify an antisense codon beginning with the third base.

```
                MetAlaIle                          Ser
Vector:         ATGGCGAT                          ATCC
                TACCGCTA                          TAGG
Insert:                5'–NXXX..................-3'
                       3'-.................YYYN2N1-5'
```

Sense primer: `5'-NXXX.....`, where XXX is the first codon of the target insert
If N = G, Met codon is generated instead of Ile.

Antisense primer: `5'-`N_1N_2`YYY .....`, where YYY is the last codon of the target insert
If N_1N_2 = CA or TA, a stop codon is generated in the sense strand.

III. Vector and Insert Preparation

A. Vector

For plasmid vector preparation, use the restriction enzyme manufacturer's recommended buffer and incubation conditions. Many combinations of enzymes are compatible when used together in the same buffer. Note that the digestion efficiency varies with the enzyme, reaction conditions and relative proximity of the restriction sites. In general, enzymes with compatible buffers and sites that 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 10 or fewer bp separates the sites, the digestions should be performed sequentially. The first digestion should be done with the enzyme that is the poorest cutter. The second enzyme should be added after the first 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.

If cloning into a single site or two blunt sites, dephosphorylate the vector following digestion to decrease the background of non-recombinants due to self-ligation of the vector. Molecular biology grade calf intestinal (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 plasmids, 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. The Novagen® SpinPrep™ Gel DNA Kit (Cat. No. 70852-3) is ideal for rapid isolation of DNA fragments from agarose gel slices.

B. Insert

Sticky ends

Inserts prepared by restriction enzyme digestion possess phosphorylated 5'-ends. Preparing inserts by restriction digestion from existing vector constructs is straightforward. Note, however, to minimize the possibility of the original vector interfering with the subsequent cloning steps, the insert should be gel purified using SpinPrep™ Gel DNA Kit (Cat. No. 70852-3).

PCR can be used to add convenient restriction enzyme sites onto the ends of target genes. 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%. Restriction sites should be flanked by 3–10 (enzyme dependent, consult restriction enzyme manufacturers for their recommendations) "spacer" nucleotides at the 5'-end to allow for efficient digestion. Following amplification, the PCR product should be purified prior to restriction enzyme digestion. Note that the small terminal fragments generated by digestion possess complementary overhangs that can participate in the subsequent ligation reaction resulting in decreased cloning efficiency. Fragments such as this (smaller than 50 bp) can be quickly and efficiently removed by precipitation in the presence of Pellet Paint® Co-Precipitant (Cat. No. 69049-3).

Blunt ends

Blunt ends are created by employing blunt-cutting restriction enzymes, filling in 5'-overhangs, polishing ("chewing back") 3'-overhangs or by amplifying PCR fragments with thermostable enzymes possessing 3'→5' proofreading activity. Vectors having blunt ends on both sides of the insertion site are typically dephosphorylated to prevent recircularization of the vector without an insert. Therefore, inserts should possess 5'-phosphorylated ends. PCR-generated inserts must be amplified with 5' phosphorylated primers or treated with polynucleotide kinase following PCR using standard conditions. The Perfectly Blunt® End Conversion Mix accomplishes 5'-phosphorylation as well as fill-in and polishing reactions all in one convenient reaction.

IV. Ligation and Transformation

Note: Clonables™ Ligation/Transformation Kit (Cat. No. 70526-3) is specifically designed for rapid cloning. It enables plating for recombinants in less than one hour (4). The kit provides a standardized set of reagents that enables convenient, dependable, high efficiency ligation and transformation with virtually any type of plasmid vector and insert. The kit features a unique, universal 2X Ligation Premix that contains ligase, buffer, and cofactors and supports rapid, optimal ligation of any type of DNA "sticky" end or blunt end. In addition, the kit contains NovaBlue Singles™ Competent Cells. For complete details see User Protocol TB233.

A. Ligation

For maximum ligation and transformation efficiency, the vector and insert DNA should be free of phenol, ethanol, salts, protein, and detergents, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or in water. Standard ligations are performed in a total volume of 10 µl and contain 50 ng (typically 0.020–0.025 pmol) of linearized, dephosphorylated vector, an equimolar to 5-fold excess of target insert, ligation buffer, ATP, DTT, and T4 DNA ligase. Assemble the reaction according to the type of ends being ligated. Blunt-end ligations are enhanced by a final concentration of 1 mM ATP in contrast to the 10 mM ATP typically used in sticky-end ligations.

B. Transformation

Use NovaBlue as the initial cloning host for pETBlue plasmids. NovaBlue enables blue/white screening, high quality plasmid preps for sequencing, and contains no source of T7 RNA polymerase, and thus is ideal for the establishment of recombinant plasmids under non-expression conditions. After the target plasmid is established and verified in NovaBlue, 1 µl of a 1:50 dilution of a miniprep (1 to 10 ng plasmid) can be easily transformed into a (DE3)pLacI expression host followed by IPTG induction.

C. Transformation protocol for experienced users

Note: See the following section for a detailed transformation protocol.

1. Thaw the required number of tubes of cells on ice and mix gently to ensure that the cells are evenly suspended.
2. Place the required number of 1.5 ml polypropylene microcentrifuge tubes on ice to pre-chill. Pipet 20 µl aliquots of cells into the pre-chilled tubes.
3. Add 1 µl DNA solution directly to the cells. Stir gently to mix.
4. Place the tubes on ice for 5 min.
5. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.
6. Place on ice for 2 min.
7. Add 80 µl room temperature SOC Medium to each tube.
8. **When using NovaBlue**, plate 5–50 µl cells directly on LB agar media containing 50 µg/ml carbenicillin, 12.5 µg/ml tetracycline, 70 µg/ml X-gal, and 80 µM IPTG.
   When using a (DE3)pLacI host strain, incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective media. Plate 5–50 µl cells on LB agar media containing 50 µg/ml carbenicillin, 34 µg/ml chloramphenicol, and 1% glucose.
9. Incubate inverted plates overnight at 37°C. Screen colonies as described in the *Screening* section.

D. Transformation—detailed protocol

Note: When selecting for the expression of β-lactamase, the antibiotic carbenicillin is recommended instead of ampicillin. Carbenicillin is less sensitive to the drop in the pH of the growth media that typically accompanies bacterial growth.

Competent cells in the standard kits are provided in 0.2 ml aliquots. The standard transformation reaction requires 20 µl cells, so each tube contains enough cells for 10 transformations.

DNA in ligation reactions containing high-quality reagents is suitable for direct addition to Novagen competent cells. Inactivation of the ligase is not required prior to transformation. For transformation, 1 µl of the ligation reaction usually yields sufficient numbers of colonies for screening.

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

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 rim of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible.
3. To mix cells, finger-flick the tube 1–3 times. *NEVER vortex competent cells.*
4. Cells can be refrozen at –70°C and used at a later date; however, transformation efficiencies may decline several-fold with each freeze-thaw cycle. To avoid multiple freeze-thaw cycles of the standard cells, dispense the cells into aliquots after the initial thaw and store the aliquots at –70°C or below. To dispense cells from the 0.2 ml stock, remove the stock tube quickly from the ice and finger-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 the immediately close the tube and replace on ice. After all of the aliquots have been dispensed, return any unused tubes to the freezer before proceeding with the transformation.

Procedure

1. Remove the appropriate number of competent cell tubes from the freezer (include on extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is immersed in ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells to see that they have thawed and gently flick the cells 1–2 times to evenly resuspend the cells.
3. Place the required number of 1.5 ml polypropylene microcentrifuge tubes on ice to pre-chill. Pipet 20 µl aliquots of cells into the pre-chilled tubes.
4. (Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Gently flick the tube 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.

   Note: Transformation efficiencies can be increased several fold by diluting the ligation reaction 5-fold with TE or water prior to adding the DNA to the cells, or by extracting the ligation reaction twice with 1:1 TE buffer phenol:CIAA (23:1 chloroform:isoamyl alcohol), once with CIAA, precipitating in the presence of sodium acetate, and resuspending in TE or water before adding the DNA to the cells.

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

   Note: This "heat stock" step is most easily accomplished if the tubes are in a rack that exposes the lower half of the tubes. Hold the rack in the water bath so that the lower halves of the tubes are submerged for 30 s, and then replace the rack on ice.

8. Place the tubes on ice for 2 min.
9. Add 80 µl room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
10. Selection for pETBlue transformants is accomplished by plating on media containing a final concentration of 50µg/ml carbenicillin (or ampicillin).
    When using NovaBlue: plate 5–50 µl cells directly on LB agar media containing 50 µg/ml carbenicillin, 12.5 µg/ml tetracycline, 70 µg/ml X-gal, and 80 µM IPTG. Tetracycline ensures that the selectable F'-containing *lacZ*ΔM15 is maintained and thus eliminates the background of non-recombinant white colonies that have lost the F'.
    When using a (DE3)pLacI host strain: incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on

selective media. Plate 5–50 µl cells n LB agar media containing 50 µg/ml carbenicillin, 34 µg/ml chloramphenicol and 1% glucose. The addition of glucose to LB agar plates and subsequent liquid medium reduces basal expression.

Note: Chloramphenicol (34 µg/ml) must be included in the plates used for pLacI hosts.

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

Note: During the outgrowth (or earlier if using NovaBlue), 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.

Important: The appropriate amount of transformation mixture to plate varies with the efficiency of the ligation and the competent cells. For recombinants in NovaBlue, expect 10^5–10^7 transformants per µg plasmid, depending on the particular insert and the ligation efficiency. When transforming (DE3)pLacI host strains with DNA from a plasmid preparation expect 10^3–10^5 transformants per µg plasmid.

> ***When using the Test Plasmid**, plate no more than 5 µl of the final NovaBlue transformation mix or plate 10 µl of (DE3)pLacI strains in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampicillin resistance gene,* bla*).*
>
> ***For blue/white screening of recombinants**, also include IPTG and X-gal in the LB agar. These can be spread on the plates and allowed to soak in for about 30 min prior to plating. Use 35 µl of 50 mg/ml X-gal in dimethyl formamide and 20 µl 100 mM IPTG (in water) per 82-mm plate. Alternatively, X-gal and IPTG can be added to the LB agar at final concentrations of 70 µg/ml and 80 µM, respectively, just prior to pouring the plates.*

11. Using a sterile bent glass rod or specialized spreader, spread until the sample is evenly distributed on the plate. If the plates are fairly dry, the sample and cushion will quickly absorb into the plate. Once the moisture is absorbed, do not continue spreading. If the plates are wet, spread until the sample is evenly distributed. Do not continue to spread until the sample and cushion have completely absorbed into the plate, as overspreading is lethal to the cells. Instead, after spreading briefly, set the plates 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.

    Note: ColiRollers™ Plating Beads (Cat. No. 71013-3) are glass beads that eliminate the use of the spreader and alcohol flame while evenly distributing cells with little possibility of damage.

12. 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). If performing blue/white screening, blue color development can be enhanced by placing the plates in a 4ºC refrigerator for a few hours after the colonies have reached the desired size.

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

V. Screening

A. Blue/white phenotype

The pETBlue™-1 and -2 Vectors provide for blue/white screening of recombinants. The plasmid encodes a functional *lacZ* α-peptide that complements the *lacZ* ω-fragment expressed by the host strain (*lacZ*ΔM15 on F' in NovaBlue). The resulting active β-galactosidase can cleave the chromogenic substrate X-gal to yield a dark blue colony phenotype. Inserts are cloned within the α-peptide open reading frame (ORF). Inserts disrupt this ORF, thereby preventing the production of functional α-peptide, which results in the white colony phenotype when plated on X-gal/IPTG indicator plates.

However, in addition to dark blue and white phenotypes, a light blue phenotype can be observed with relatively high frequency in vector plus insert ligations. We have found that more than 90% of these light blue colonies generated from vector plus insert ligations contain inserts of the expected size. Also note that the white colonies may develop a light blue center or "bullseye" appearance when grown to large sizes or during prolonged storage at 4°C. Presumably a small amount of functional α-peptide is produced in these recombinants by means of ribosomal frameshifting, second site translational initiation, or as an α-peptide fusion protein. The very small number of light blue or white colonies that arise from self-ligated vector appear to be the result of religating vector ends that may have been damaged during the vector preparation.

B. Colony PCR

If the cloning was successful, there are usually many more colonies produced from ligation in the presence of the insert than with the negative control. Colonies can be screened for inserts without the need for plasmid minipreps by colony PCR. To verify the presence of an insert, 5 pmol (1 µl) of one of the vector-specific primers can be used with 5 pmol of any insert-specific primer (if available and annealing conditions are compatible). Alternatively, just the vector-specific primer can be used if insert orientation information is not desired.

In addition, it is possible to quickly assess the ability of an individual clone to express a target protein *in vitro* by amplifying a colony with appropriate primers and using the DNA product as template for *in vitro* transcription and translation using the Novagen Single Tube Protein® System 3 (STP3®) T7, or EcoPro™ T7 System. Upstream primers that provide a "spacer" region before the T7 promoter allow efficient transcription of the PCR products with T7 RNA polymerase. This method allows rapid testing of clones for potential mutations, such as those that insert a stop codon (nonsense mutations). Note that the vector-insert ligation reactions can also be used as templates to generate PCR products suitable for transcription/translation.

For *in vitro* transcription/translation the upstream primer requires a minimum of 6 bp upstream of the T7 promoter sequence to enable efficient transcription by T7 RNA polymerase. The pETBlueT7UP Primer and pETBlueDOWN Primer are appropriate upstream and downstream primers for *in vitro* transcription/translation analysis or for colony screening of pETBlue-1 and pETBlue-2 vectors. The T7 Promoter Primer and pETBlueUP Primer are appropriate upstream primers for colony screening only. The recommended downstream primers for colony screening of the pETBlue vectors are the pETBlueDOWN or T7 Terminator Primers. The sequence and binding location for the primers is indicated on the respective vector maps. Sequence details can also be found at www.merck4biosciences.com.

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 collect as many cells as possible. If a copy of the colony is desired, touch the pipet tip to a plate before transferring the bulk of the colony to the tube in the next step.
2. Transfer the bacteria to a 0.5 ml tube containing 50 µl 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. Place on ice until use.
6. Make a master reaction mix on ice using the following amounts per reaction. To account for pipetting losses, it is convenient to multiply the amounts by X.5, where X is the number of reactions.
   Per reaction:

31.75 µl	PCR-grade Water
1 µl	dNTPs (10 mM each dATP, dCTP, dGTP, and dTTP)
1 µl	upstream primer, 5 pmol/µl
1 µl	downstream primer, 5 pmol/µl
5 µl	10X Nova*Taq* Buffer with $MgCl_2$
0.25 µl	Nova*Taq*™ DNA Polymerase (1.25 U)
40 µl	total volume

   *Note: If using the Nova*Taq *Buffer without $MgCl_2$, compensate by adding $MgCl_2$ to a final concentration of 1.5–2.5 mM and decreasing the volume of water.*
7. Mix gently and, if necessary centrifuge briefly. Add 40 µl of the master mix to each sample, mix gently, overlay 2 drops mineral oil, cap the tubes, and place the samples in a thermal cycler.

   *Note: As an optional step, a hot start procedure can be used in which the cell lysate samples are warmed to 80°C before the addition of the master mix. Alternatively, use Nova*Taq *Hot Start DNA Polymerase. For greatest accuracy, specificity, and yield of long complex targets use KOD HiFi, KOD Hot Start, and KOD XL DNA Polymerases, respectively.*
8. Process in the thermal cycler for 35 cycles, as follows:

Denature	1 min at 94°C
Anneal	1 min at the proper annealing temperature (usually 55°C for vector primers)
Extend	2 min at 72°C

   Repeat for 35 cycles
   Final extension 5 min at 72°C
9. To remove the oil overlay and inactivate the polymerase, add 100 µl chloroform, mix 30 s, and centrifuge for 1 min. The top aqueous phase (which may appear cloudy) contains the DNA products. Transfer the aqueous phase to a fresh tube. If desired, remove a 5–10 µl sample for gel analysis. Store the remainder at –20°C.
10. If an appropriate PCR primer combination was used, 2 µl of the PCR product can be added directly to a Single Tube Protein® System 3, T7 reaction for protein synthesis.
11. 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 3M sodium acetate and 115 µl 95% ethanol. 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 50 µl deionized water. The addition of Pellet Paint® Co-Precipitant to the DNA facilitates recovery in the precipitation step without affecting performance in the EcoPro reaction. Use 2–4 µl in the EcoPro T7 reaction.

C. DNA isolation and sequencing

After positive clones are identified, plasmid DNA can be isolated for transformation into expression hosts, restriction mapping, and sequence analysis. Plasmid DNA from candidate recombinants can also be evaluated using *in vitro* transcription/translation analysis. It is important that the template be RNase-free for *in vitro* transcription and translation. The pETBlue™ vectors contain the high copy pUC origin of replication and produce DNA yields similar to other pUC-based plasmids. Purify Plasmid DNA isolated using protocols from scientific literature. The plasmid DNA must be RNase-free, and thus may require an additional phenol:CIAA extraction to eliminate RNases. A satisfactory procedure is to add TE to 100 µl, and then extract successively with 1 vol TE-buffered phenol, 1 vol phenol:CIAA (1:1; CIAA is chloroform:isoamyl alcohol, 24:1), and 1 vol CIAA. Transfer the final aqueous phase to a fresh tube and

add 0.1 vol 3M Na acetate and 2 vol 100% ethanol. Mix and place at –20°C for 30 min, spin 5 min at 14,000 × 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 with the TE buffer before extraction to facilitate recovery of the DNA (the –20°C incubation can be eliminated if using Pellet Paint Co-Precipitant).

Because pETBlue vectors contain an f1 origin of replication, it is also possible to prepare single stranded plasmid DNA from the NovaBlue strain by infection with a single stranded DNA helper phage. The f1 origin in pET Blue 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.

VI. Target Protein Expression

Target protein expression can be induced from appropriately cloned pETBlue™ recombinants by induction of the T7*lac* promoter. Induction can be achieved in the indicated host strains in the following ways.

A. Induction in NovaBlue by λCE6 infection

Expression can be induced from the NovaBlue host used for blue/white screening by infection with the Bacteriophage λCE6. The λp_L and λp_I promoters control the expression of T7 gene *1* in this phage. Therefore, T7 RNA polymerase is immediately produced in host cells upon CE6 infection. This in turn results in transcription of the target gene from the T7*lac* promoter present in the pETBlue plasmids. T7 RNA polymerase transcribes target DNA so actively that normal CE6 phage development cannot proceed. Because no T7 RNA polymerase is present in the cell before infection, 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 (Cat. No. 69390-3) and is described in User Protocol TB007.

B. Induction in (DE3)pLacI expression hosts

pETBlue recombinants identified and isolated from the blue/white screening host NovaBlue can be transformed into specialized (DE3)pLacI expression hosts for IPTG-based induction. These hosts carry a chromosomal copy of T7 RNA polymerase under the control of the *lacUV5* promoter and supply sufficient *lac* repressor from the compatible pLacI plasmid to ensure stringent repression in the uninduced state. pLacI encodes the *lacI* gene and chloramphenicol resistance marker on a plasmid that is compatible with pETBlue. Unlike other T7*lac* promoter-based pET vectors, pETBlue plasmids do not contain the *lac* repressor gene and therefore additional *lac* repressor is required. Expression host strains compatible with pETBlue (see table on following page) are appropriate for IPTG-based induction.

(DE3)pLacI Expression Strains				Competent Cells	
Strain	**Resistance[1]**	**Derivation**	**Key Feature(s)**	**Size**	**Cat. No.**
Origami 2(DE3)pLacI	Tet + Str[2] + Cam	K-12	Kan sensitive; *trxB*/*gor* mutant, greatly facilitates cytoplasmic disulfide bond formation; Leu auxotroph	0.4 ml 1 ml	71347-3 71347-4
Origami B(DE3)pLacI	Kan + Tet + Str[2] + Cam	Tuner™ (B strain)	*trxB*/*gor* mutant; greatly facilitates cytoplasmic disulfide bond formation BL21 *lacZY* deletion mutant; allows precise control with IPTG	0.4 ml 1 ml	70838-3 70838-4
Rosetta™(DE3)pLacI	Cam	BL21	Expresses six rare tRNAs; facilitates expression of genes that encode rare *E. coli* codons	0.4 ml 1 ml	70920-3 70920-4
Rosetta 2(DE3)pLacI	Cam	BL21	Expresses seven rare tRNAs; facilitates expression of genes that encode rare *E. coli* codons	0.4 ml 1 ml	71404-3 71404-4
RosettaBlue™(DE3)pLacI	Tet + Cam	NovaBlue (K-12)	Expresses six rare tRNAs; facilitates expression of genes that encode rare *E. coli* codons *recA*$^-$, *endA*$^-$, *lacI*^{q}; high transformation efficiency	0.4 ml 1 ml	71060-3 71060-4
Rosetta-gami 2(DE3)pLacI	Tet + Str[2] + Cam	Origami 2 (K-12)	Expresses seven rare tRNAs; facilitates expression of genes that encode rare *E. coli* codons Kan sensitive; *trxB*/*gor* mutant; greatly facilitates cytoplasmic disulfide bond formation; Leu auxotroph	0.4 ml 1 ml	71353-3 71353-4
Rosetta-gami B(DE3)pLacI	Kan + Tet + Str[2] + Cam	Origami B (B strain)	Expresses six rare tRNAs; facilitates expression of genes that encode rare *E. coli* codons *trxB*/*gor* mutant; greatly facilitates cytoplasmic disulfide bond formation BL21 *lacZY* deletion mutant; allows precise control with IPTG	0.4 ml 1 ml	71138-3 71138-4
Tuner™(DE3)pLacI	Cam	BL21	BL21 *lacZY* deletion mutant; allows precise control with IPTG	0.4 ml 1 ml	70625-3 70625-4

1. The Resistance column in the table refers to selectable marker(s) possessed by the strain in the absence of target plasmids. Appropriate concentrations for selection are as follows:
   Cam: 34 µg/ml chloramphenicol; Kan: 15 µg/ml kanamycin; Str: 50 µg/ml streptomycin; Tet: 12.5 µg/ml tetracycline

2. These strains carry a mutation in ribosomal protein (*rpsL*) conferring resistance to streptomycin; therefore streptomycin is no necessary to maintain strain genotype. If using pCDF vectors, spectinomycin must be used for antibiotic selection because rpsL mutation confers streptomycin resistance.

C. Induction and analysis

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. For this purpose 50 ml cultures (as presented below) are convenient, but the analysis can be scaled accordingly.

The following protocol is designed to do the following:

- Verify that the target protein is produced upon induction
- Determine whether the target protein is present in the soluble or insoluble fraction.
- Verify the presence of detection tags if any in the target protein

To facilitate the gel and Western analysis, two worksheets are provided to record data and calculate normalized loading volumes for standard mini gels (page 20). If larger gels are used, the loading volumes should be scaled up accordingly. This formula relies on the generation of accurate OD_{600} readings at harvest and the determination of concentration factors for the fractions generated. The concentration factor represents the volume of original culture used to produce the fraction divided by the final volume of the fraction.

Note To prevent elevated basal expression levels and depletion of the antibiotic from the medium, avoid overnight cultures (see page 28).

Growth and induction

1. Prepare a starter culture of the pETBlue™ recombinant in a (DE3)pLacI expression host strain as follows: inoculate 3 ml of appropriate media plus 0.5–1% glucose plus antibiotic(s) in a culture tube with a sterile loop of cells taken from a plate or appropriately prepared glycerol stock (see page 28).

   Note: See Appendix section "Toxic genes and plasmid instability" for the rationale concerning the inclusion of glucose in the growth media.

2. Incubate at 37°C with shaking at 250 rpm to an OD_{600} of approximately 0.6–1.0. Store the culture at 4°C overnight. The following morning, collect the cells by centrifugation. Resuspend the cells in 3 ml fresh medium plus 0.5–1% glucose plus antibiotic(s) and use this to inoculate 100 ml medium plus 0.5–1% glucose plus antibiotic(s).
3. Shake the 100 ml culture at the desired temperature until the OD_{600} is approximately 0.5–1.0 (e.g., 2–3 h in LB broth, 37°C). Monitor the OD_{600} during growth by removing samples aseptically.
4. Just prior to induction, split the 100 ml culture into 2 x 50 ml cultures. For full induction add IPTG to 1 mM (500 µl of sterile 100 mM IPTG) to one of the 50 ml cultures. The other culture will serve as the uninduced control. Incubate with shaking at the desired temperature for the appropriate amount of time, typically 2–4 h when performing a standard 37°C induction.

   Note: When using lacZY*mutant strains [i.e., Tuner™(DE3)pLacI and derivatives] altering IPTG concentration in the range of 25 µM to 300 µM IPTG should yield target protein expression that ranges from barely detectable to nearly fully induced in 3 h at 37°C.*

Optical density analysis of the induced culture

1. After induction and just prior to harvest, shake well to ensure a homogeneous suspension and remove 0.5–1 ml samples of the induced and uninduced cultures.
2. Determine the OD_{600} of the culture as accurately as possible by diluting the sample in the same medium 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). Adjust the spectrophotometer to zero with the same medium used for growth.
3. Record both the dilution factor and the OD_{600} reading on the attached worksheet (page 20).

Cell lysis and fractionation using BugBuster® Protein Extraction Reagent and Benzonase® Nuclease

BugBuster Protein Extraction Reagent is a simple, rapid, non-mechanical method to gently disrupt the cell wall of *E. coli* resulting in the liberation of soluble protein. It provides an alternative to mechanical methods such as French Press or sonication. 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. At this point, rLysozyme™ Solution can be added to increase the efficiency of cell lysis and protein extraction, and Benzonase Nuclease (a genetically engineered endonuclease) can be added to reduce lysate viscosity for increased flow rates during purification. During a brief incubation, soluble proteins are released and nucleic acids digested. Following clarification by centrifugation, the low viscosity extract, containing soluble protein is ready to load on any of the affinity chromatography resins offered by Novagen. The insoluble fraction can be further processed to yield purified inclusion bodies. Other formulations of BugBuster are available and include 10X BugBuster (concentrated BugBuster solution), BugBuster (primary amine-free) that consists of BugBuster in PIPPS buffer, and BugBuster HT (a ready to use mixture of BugBuster and Benzonase). See User Protocol TB245 for more information.

General considerations

- BugBuster and Benzonase are most efficient when used at room temperature. Storage of BugBuster at temperatures below 4°C may cause precipitation of the detergents. Incubate in a room temperature water bath with gentle swirling to redissolve.
- BugBuster can be used on fresh or frozen cell pellets. For comparisons of multiple samples (e.g., extended time course analysis) all cell pellets should be processed identically (all fresh or all frozen). Superior extraction efficiencies can be obtained by freezing the cell pellet prior to resuspension in BugBuster Protein Extraction Reagent, however some target proteins may be inactivated by freeze-thaw cycles.

  Tip: For optimal extraction especially of high molecular weight proteins (> 70 kDa), addition of rLysozyme™ Solution (Cat. No. 71110) and/or freezing of bacterial cell pellets prior to BugBuster extraction is highly recommended.

  - Extraction efficiency is somewhat strain-dependent and appears to be especially efficient with the BL21 strain and derivatives.
  - BugBuster is compatible with Tris and phosphate-based buffer systems in the near-neutral pH range. The detergents in BugBuster will precipitate at or above 1 M NaCl. Evaluate extraction on a small scale when using high salt buffers or acid or alkaline pH ranges for chromatography. BugBuster is fully compatible with all Novagen chromatography resins.
  - BugBuster reagent is compatible with reducing agents such as Tris(hydroxypropyl)phosphine, 2-mercaptoethanol, and DTT as well as with EDTA. Note that reducing agents may activate proteases and EDTA will interfere with protein binding to His•Bind® Resin. Up to 20 mM 2-mercaptoethanol can be used with Ni-NTA His•Bind Resin.
  - Benzonase can be diluted in 50 mM Tris-HCl, 20 mM NaCl, 2 mM $MgCl_2$, pH 8.0 for handling small quantities and can be stored at 4°C for several days without loss of activity.
  - Benzonase is not recommended for nuclease-free preparations. Depending on the protein purification method, Benzonase may be removed. Residual nuclease activity can be monitored by incubation of the purified protein with RNA or DNA markers followed by gel analysis.
  - Benzonase is inhibited (approximately 50% reduction in relative activity) by monovalent cation concentrations > 50 mm, phosphate concentrations > 20 mM, and by ammonium sulfate concentrations > 25 mM.
  - If the target protein requires a reducing environment, 0.5 M THP Solution (Cat. No. 71194) can be used. THP [Tris(hydroxypropyl)phosphine] is a water soluble, odorless, ready-to-use, neutral reducing agent that is more stable and effective than DTT as a sulfhydryl reductant. THP is more resistant to air oxidation than DTT and is compatible at a concentration of 1.0 mM for use in immobilized metal affinity chromatography with His•Bind and Ni-NTA His•Bind Resins.
  - For SDS-PAGE and Western blot analysis, a load volume of approximately 2.4 µl should give a normalized amount of protein, given a concentration factor of 25 and an OD_{600} of 3.0 at harvest, using a 15-well mini gel. When 1.5 culture volumes of BugBuster® Protein Extraction Reagent is used, this would correspond to a load volume of approximately 12 µl (since the concentration factor is 5). Because the optimal amount of material to load will vary with the expression level of the target protein, the efficiency of the extraction, and detection sensitivity of the Western blot method, these amounts should be used as guidelines only.

Soluble fraction

This fraction will consist of soluble protein present in both the periplasm and cytoplasm. If a separate periplasmic fraction is desired, first follow the osmotic shock procedure in the pET System Manual (User Protocol TB055). The pellet from that procedure can be used in this protocol.

1. Harvest cells from liquid culture by centrifugation at 10,000 × g for 10 min using a weighed centrifuge tube. For small scale extractions, (1.5 ml or less) centrifugation can be performed in a 1.5 ml tube at 14,000–16,000 × g. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.
2. Resuspend the cell pellet in room temperature BugBuster reagent by pipetting or gentle vortexing, using 5 ml reagent per gram of wet cell paste. This typically corresponds to about 2.5 ml per 50 ml culture. For small cultures use up to 1/5 culture volume for resuspension (e.g., use 300 µl BugBuster for 1.5 ml cultures). There are no adverse effects to using larger volumes of BugBuster® Protein Extraction Reagent.
   Optional:
   a) Add 1 µl (25 units) of Benzonase® Nuclease per ml of BugBuster reagent used for resuspension. Although Benzonase requires Mg^{2+} for activation, Mg^{2+} is not required for viscosity reduction or nucleic acid digestion under the conditions described here.
   b) Add 1 KU rLysozyme™ Solution per ml BugBuster reagent (5 KU/g cell paste). rLysoyzme solution can be diluted using 100 mM NaCl, 50 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 0.1% Triton® X-100, pH 7.5. Dilutions should be used as soon as possible and stored on ice or at 4°C until use. Do not store diluted rLysozyme Solution at -20°C, as freezing may result in loss of activity.

      Note: Alternatively, Lysonase™ Bioprocessing Reagent (Cat. No. 71230), an optimized ready-to-use mixture of rLysozyme Solution and Benzonase Nuclease, can be used instead of separate additions of Benzonase and rLysozyme. For efficient protein extraction with BugBuster use 10 µl Lysonase per gram wet cell paste.

   c) Add protease inhibitors. Protease inhibitors are compatible with BugBuster and Benzonase.
   d) Benzonase and rLysozyme can be pre-mixed with BugBuster for rapid sample processing. Pre-mixed Benzonase, rLysozyme, and BugBuster should be prepared immediately before use.
3. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature. If Benzonase was added, the extract should not be viscous at the end of the incubation.
4. Remove insoluble cell debris by centrifugation at 16,000 x g for 20 min at 4°C. If desired, save the pellet for inclusion body purification as described below.
5. Transfer the supernatant to a fresh tube. The soluble extract can be loaded directly onto any Novagen purification resin (and numerous other systems). Maintain clarified extracts on ice for short term storage (2–3 h) or freeze at –20°C until needed. Extracts should be stored at a temperature compatible with target protein activity; some target proteins may be inactivated by freeze-thaw cycles.

Inclusion body purification

1. Process the induced culture according to steps 1–4 above for the soluble protein fraction.
2. Resuspend the pellet from step 4 above in the same volume of BugBuster® Protein Extraction 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.
3. Add rLysozyme™ Solution to a final concentration of 1 KU/ml. Mix by gently vortexing and incubate at room temperature for 5 min.
4. Add 6 volumes of 1:10 diluted BugBuster reagent (in deionized water) to the suspension and mix by vortexing for 1 min.
5. Centrifuge the suspension at 5,000 × g for 15 min at 4°C to collect the inclusion bodies. Remove the supernatant with a pipet.
6. Resuspend the inclusion bodies in 0.5 the original culture volume of 1:10 diluted BugBuster, mix by vortexing, and centrifuge as in step 5 twice. Resuspend once more but centrifuge at 16,000 × g for 15 min at 4°C and remove the supernatant.
7. Resuspend the final pellet of purified inclusion bodies in the buffer of your choice, preferably with a buffer compatible with the desired purification method. The final pellet of inclusion bodies is compatible with resuspension in 1X Solubilization Buffer provided in the Novagen Protein Refolding Kit (see User Protocol TB234) or other denaturing buffers.

Cell lysis and fractionation, traditional protocol

Total cell 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 2X Sample Buffer (2X SB = 100 mM DTT, 80 mM Tris-HCl, 15% glycerol, 2% SDS, 0.006% bromophenol blue, pH 6.8) and sonicate with a microtip at the following settings: power level between 2–3, at 20–30% duty for 8–10 bursts (Branson Sonifier® 450; sonication conditions may vary with the equipment). Alternatively, pass the sample through a 27-gauge needle several times to reduce the viscosity.
4. Heat for 3 min at 85°C to denature the proteins and then store at –20°C until SDS-PAGE analysis.

Soluble fraction

Crude soluble and insoluble fractions can be prepared by the following protocol.

1. Harvest 40 ml of the culture by centrifugation at 10,000 x g for 10 min at 4°C. 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.
2. Completely lyse the cells by one of the following methods:
   a) *French Press*. Perform two passes at 20,000 psi using a chilled pressure cell.
   b) *Lysozyme treatment plus sonication*. Mix by swirling and sonicate on ice using a microtip with the power level set between 4–5, at 40–50% duty for 15–20 bursts. It is important to keep the sample cold during sonication to avoid heat denaturation of proteins. The above settings are general recommendations and may need to be adjusted depending on the energy output of a given sonicator.
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.

   Note: rLysozyme Solution (Cat. No.71110-3) is available for efficient bacterial cell lysis.
4. Spin the entire lysate of 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. Take a 1.5-ml sample of the lysate and centrifuge at 14,000 x g for 10 min to separate the soluble and insoluble fractions. Transfer 100 µl of the soluble supernatant to a new tube. Remove and save the remaining supernatant for activity assays, as desired. Save the insoluble pellet fraction on ice for processing, as described in the next section.
6. Add 100 µl of 2X SB to the 100 µl soluble fraction sample. Heat for 3 min at 85°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

Insoluble fraction

The insoluble fraction (inclusion bodies) can be further purified by repeated centrifugation and washing steps. However, the product will be contaminated at some level with other proteins and nucleic acids. In some cases, purified inclusion bodies are suitable for direct use as antigens for the preparation of antibodies against the target protein (5). 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 can be released into the soluble fraction by including a detergent during lysis.

1. Wash the insoluble pellet by resuspending in 750 µl of 20 mM Tris-HCl, pH 7.5. Centrifuge at 10,000 x g for 5 min, remove the supernatant, and repeat the wash step.
2. Resuspend the final pellet in 1.5 ml of 1% SDS with heating and vigorous mixing or sonication if necessary (resuspension in this volume maintains the concentration factor at 10X).
3. Take a 100 µl sample and add it to 100 µl of 2X SB. Heat for 3 min at 85°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

D. SDS-PAGE and Western blot analysis

Normalize the sample 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.

Normalized SDS-PAGE gel loading

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

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

Worksheet 2: Determination of the normalized volume of sample to load on a standard 10-well or 15-well mini SDS-PAGE gel. 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 a 1 ml 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 (SCF)	**OD_{600} at harvest**	**Z ($SCF \times OD_{600}$)**	**180 µl ÷ Z**	**270 µl ÷ Z**
Induced Samples					
TCP					
Soluble Cytoplasmic					
Insoluble Fraction					
Uninduced Samples					
TCP					
Soluble Cytoplasmic					
Insoluble Fraction					

The identity of the target protein can be determined by Western blot analysis with a target protein-specific antibody or detection based on pETBlue™-2 fusion partners, the HSV•Tag® or His•Tag® sequences. For Western blots dilute the samples 1:25 to 1:50 in 1X SB and load along with Perfect Protein™ (Cat. No. 69959-3) or Trail Mix™ (Cat. No. 70982-3) Western Markers. Because the markers carry the S•Tag™ and His•Tag sequences, they can be detected by either the S-protein AP (Cat. No. 69598-3) or HRP (Cat. No. 69047-3) Conjugates (6) or the His•Tag Monoclonal Antibody (Cat. No. 70796-3), as appropriate. Perfect Protein and Trail Mix Western Markers will display bands at 15, 25, 35, 50, 75, 100, 150, and 225 kDa. Trail Mix Markers also include three prestained proteins at 100, 16, and 15 kDa to allow direct visualization of protein mobility during electrophoresis.

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

VII. Purifying Target Proteins

The methods selected for protein purification depend on many variables, including the properties of the protein of interest, its location and form within the cells, the pETBlue™ 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. One advantage of the pETBlue 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.).

Proteins expressed in pETBlue-2 with a C-terminal His•Tag® sequence can be purified using Ni-NTA His•Bind® or His•Bind Resins. For a listing of His•Bind Resins, see the following section.

A. Purification tools

A brief description of products for extract preparation and affinity chromatography is indicated below. For detailed information, see the indicated User Protocol available at www.merck4biosciences.com.

Extraction reagents	**Cat. No.**	**Size**	**User Protocol No./Capacity and features**
BugBuster® Protein Extraction Reagent	70584-3 70584-4	100 ml 500 ml	TB245; Use 5 ml/g wet cell paste. Tris-buffered.
BugBuster 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 ml of culture volume with PopCulture Reagent and 1 KU per ml of 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
Lysonase™ Bioprocessing Reagent	71230-3 71230-4 71230-5	0.2 ml 1 ml 5 ×1 ml	TB361; Optimized blend of rLysozyme Solution and Benzonase Nuclease. Use 3 µl per ml lysis buffer
His•Tag® purification	**Cat. No.**	**Size**	**User Protocol 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

(continued on next page)

His•Tag® purification (continued)	Cat. No.	Size	User Protocol No./Capacity and Features
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•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 Purification Kit	70239-3		TB054; 10 ml His•Bind Resin, Buffers and Chromatography Columns
BugBuster® Ni-NTA His•Bind Purification Kit	70751-3		TB273; 10 ml Ni-NTA His•Bind Resin, BugBuster, Benzonase, and Chromatography Columns
BugBuster His•Bind Purification Kit	70793-3		TB054; 10 ml His•Bind Resin and Buffer, BugBuster, Benzonase, and Chromatography Columns
PopCulture® His•Mag Purification Kit	71114-3		TB054; Process 40 × 3-ml cultures purifying up to 375 µg per 3 ml culture
RoboPop™ His•Mag Purification Kit	71103-3		TB327; Purify up to 12 mg per 96 wells
RoboPop Ni-NTA His•Bind Kit	71188-3		TB346; Purify up to 96 mg per 96 wells

B. Solubilization of inclusion bodies and refolding proteins

A variety of methods have been published describing refolding of insoluble proteins (7–12). 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.

The Novagen Protein Refolding Kit (Cat. No. 70123-3) uses a CAPS buffer at alkaline pH in combination with N-lauroylsarcosine to achieve solubility of the inclusion bodies, followed by dialysis in the presence of DTT to promote refolding. A discussion of various methods and factors involved in protein solubilization and refolding are included in User Protocol TB234, available at www.merck4biosciences.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. Refolded fusion proteins can be affinity purified under native conditions using His•Tag and other appropriate affinity tags.

VIII. References

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IX. Appendix A: Optimizing Expression

The following sections describe procedures and recommendations regarding optimizing expression of your target protein. Included are considerations for plasmid stability, protein solubility, and a discussion of factors that influence target gene expression.

A. Solubility

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 suggested methods 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 an increase or decrease in the proportion of soluble and insoluble forms.

Temperature

Growth at 37°C causes some proteins to accumulate as inclusion bodies, while incubations at 30°C lead to soluble, active protein (13). In some cases, prolonged (e.g., overnight) induction at lower temperatures (15–20°C) may prove optimal for the yield of soluble proteins.

Lysis buffer

The portioning 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® Bind 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 can 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, when used in combination with rLysozyme™ Solution, can be an effective choice to consider for solubilization. The proprietary formulation of BugBuster and PopCulture utilizes 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 proteins and it is possible many may not be solubilized. Selection of 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” (14). Note, however, that the addition of detergent may affect downstream applications.

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, it can be dissociated by adding salt to the lysis buffer or digesting the nucleic acid with a nuclease such as Benzonase® Nuclease (see User Protocol TB261).

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™(DE3)pLacI, Origami 2(DE3)pLacI, Origami B(DE3)pLacI, Rosetta-gami™(DE3)pLacI, Rosetta-gami 2(DE3)pLacI, or Rosetta-gami B(DE3)pLacI 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*) and glutathione reductase *(gor*) mutations found in these strains increase disulfide bond formation in the cytoplasm (15, 16). The Origami 2(DE3)pLacI and Rosetta-gami 2(DE3)pLacI strains contain the same *trxB/gor* mutations as the original strains, but are also kanamycin sensitive. If the target protein contains disulfide bonds and the insert encodes rare codons, the Rosetta-gami strains may provide an optimal environment (see below, *Correcting for rare codon usage*).

The Tuner™(DE3)pLacI strain and its derivatives [Origami™ B(DE3)pLacI and Rosetta-gami™ B(DE3)pLacI] 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. 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 (17, 18) as well as truncation products. These effects seem to be most severe when multiple rare codons occur near the amino terminus (19). A number of studies have indicated that high usage of Arg codons AGA and AGG can have severe effects on protein yield. The impact appears to be highest when these codons are present near the N-terminus and when they appear consecutively (20, 22). 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 (20). Increasing other rare tRNAs for AUA, CUA, CCC, or GGA have all been used to augment the yield and fidelity of heterologous proteins (23).

The Rosetta™ strains are designed to enhance expression of target protein that contain rare codons. The original Rosetta strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, and GGA on a compatible chloramphenicol-resistant plasmid, pRARE (24). The Rosetta 2 strains supply a seventh rare codon (CGG) in addition to the six found in the original Rosetta strains (25). The Rosetta strains containing pLacI have the rare codon tRNAs on the same backbone as the *lac* repressor. The Rosetta-gami™ B(DE3)pLacI strain combines the key features of BL21 (and its Tuner™ derivative), Origami™, and Rosetta™ to enhance both the expression of eukaryotic proteins and the formation of target protein disulfide bonds in the bacterial cytoplasm. RosettaBlue™ (DE3)pLacI, Rosetta-gami(DE3)pLacI, and Rosetta™-gami 2(DE3)pLacI strains are derived from and contain the features of the corresponding K-12 NovaBlue, Origami, and Origami 2 strains, respectively. RosettaBlue(DE3)pLacI has the added benefits of high transformation efficiency and stringency due to high level of *lac* repressor ($lacI^q$). The Rosetta-gami(DE3)pLacI strain is *trxB/gor* mutant enhancing protein folding through the formation of disulfide bonds. The Rosetta-gami 2(DE3)pLacI strain supplies seven rare codons, has the same *trxB/gor* mutations as the original Rosetta-gami strain, and is also kanamycin sensitive.

C. Toxic genes and plasmid instability

In contrast to the wild type *lac* promoter, the *lacUV5* promoter that controls expression of T7 RNA polymerase in λDE3 lysogens is relatively insensitive to cAMP/CRP stimulation (26). However, more recently it has been demonstrated that when λDE3 hosts are grown to stationary phase in media lacking glucose, cAMP-mediated derepression of the wild type and *lacUV5* promoters occurs (27). Although growing λDE3 hosts to stationary phase is not recommended, derepression can be effectively avoided in overnight (16 h) cultures by growing λDE3 hosts in media containing 0.5–1.0% glucose, which inhibits cAMP production(28). Typically, glucose addition is not required to maintain plasmid stability; however, in cases of extremely toxic target genes the addition of glucose to agar plates and liquid media should help decrease basal expression. Note that addition of glucose is neither necessary nor recommended during the cloning steps in non-expression hosts.

pETBlue™ plasmids are relatively stable and are retained by a high fraction of host cells even after growth for several 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 (29). The most toxic gene products may be stabilized and expressed in this system (see User Protocol TB333).

In the pETBlue 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.

Use of ampicillin

Use of ampicillin as a selective antibiotic requires special care, because β-lactamase is produced in substantial amounts and is secreted into the medium, where it can destroy all of the ampicillin. In addition, ampicillin is susceptible to hydrolysis under the acidic media conditions created by bacterial metabolism. This means that a culture in which the cells carry an unstable plasmid will be growing under ampicillin selection only until enough β-lactamase has been secreted to destroy the ampicillin in the medium. From that point on, cells that lack plasmid will not be killed and will begin to overgrow the culture. For a typical 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.

D. Maximizing expression

Simple precautions are recommended to maximize retention of plasmid through the procedures for isolating, maintaining, and expressing target plasmids. Experiments at Novagen have shown that the use of carbenicillin in place of ampicillin helps prevent overgrowth of cultures by cells that have lost the plasmid, partially due to its superior stability at low pH.

The following protocol usually produces the highest possible fraction of expression host cells retaining pETBlue recombinant ampicillin-resistant plasmid.

Storage of ampicillin-resistant pETBlue expression host strains

1. Inoculate a colony from the expression host transformation plate into 2 ml LB plus 0.5–1% glucose plus 50 µg/ml carbenicillin plus appropriate host strain antibiotics. Incubate for a few hours, until the culture becomes slightly turbid.
2. Streak a sample on a LB agar plate containing carbenicillin plus appropriate host strain antibiotics to obtain a single colony.
3. As soon as the colony develops (usually overnight at 37°C), inoculate into 2 ml LB plus 0.5–1% glucose plus 50 µg/ml carbenicillin plus appropriate host strain antibiotics and grow until OD_{600} is approximately 0.5.

   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.
4. Mix 0.9 ml of culture with 0.1 ml of 80% glycerol in a cryovial and store in a –70°C freezer.

E. Difficult target proteins

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

The target protein itself may interfere with gene expression or with the integrity of the cell. Sometimes pulse labeling shows a gradual or rapid decrease in the rate of protein synthesis as target protein accumulates, or sometimes all protein synthesis stops before any target protein can be detected. Occasionally, considerable lysis of a culture is observed.

Plasmids containing extremely toxic genes may be destabilized in λDE3 lysogens by cAMP-mediated derepression of the *lacUV5* promoter, which may increase basal expression of T7 RNA polymerase (28). This derepression can be effectively delayed by including 0.5–1% glucose in the culture medium.

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 seemed to accumulate. Instability of certain target proteins might also be expected, although BL21 and its derivatives (e.g., Tuner™, Origami™ B, Rosetta™, and Rosetta-gami™ B strains) are deficient in the *lon* and *ompT* proteases and many proteins produced in these strains are quite stable. Some relatively short proteins produced by out-of-frame fusions are also quite stable in these strains, whereas others are so rapidly degraded they remain undetected by pulse labeling.

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 (30, 31). In practice, little or no processing was observed by these authors when the following amino acids occupied the penultimate position: His, Gln, Glu, Phe, Met, Lys, Tyr, Trp, or Arg. Processing ranged from 16%–97% when the remaining amino acids occupied this position.

The relationship between the amino terminal amino acid of a protein and the stability of the protein in bacteria has been determined and is called the N-end rule (32). These authors reported protein half-lives of only 2 minutes when the following amino acids were present at the amino terminus: Arg, Lys, Phe, Leu, Trp, or Tyr. In contrast, all other amino acids conferred half-lives of > 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.

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 (33, 34). 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 affinity tags at both ends of the target protein.

F. Other factors influencing expression level

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

1. Secondary structure in the mRNA transcript can interfere with the AUG translation initiation codon and/or the ribosome binding site (35–37).
2. 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 EcoPro™ T7 System (User Protocol TB278) or Single Tube Protein® System 3, T7 (User Protocol TB206).