TALON® Express Bacterial Expression & Purification Kit User Manual

Cat. Nos. 635639 & 635640
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I. Introduction & Protocol Overview

TALON® Express Bacterial Expression & Purification Kits are designed for the cloning, expression, and purification of polyhistidine-tagged proteins using *E. coli*. Bacterial expression is typically the first approach to the production of proteins for *in vitro* studies, due to its simplicity and robustness. However, since the physicochemical properties of proteins vary greatly, some of the steps required for protein production may be difficult to perform successfully for certain proteins. These include the cloning of a gene of interest into an expression vector, expression of the gene of interest, and purification of the corresponding protein (see Figure 1 and Table I). Our TALON® Express Bacterial Expression and Purification Kits have been designed to expedite the use of bacterial expression by incorporating a number of technologies that increase the efficiency of each of these steps. This system, which is based on the inducible T7 expression system (pET) developed by F. William Studier and colleagues at Brookhaven National Laboratories (Moffatt & Studier, 1986, Rosenberg *et al.*, 1987, Studier *et al.*, 1990), contains IPTG-inducible, pET-based vectors providing high levels of protein expression.

Choice of cloning options

TALON Express Bacterial Expression and Purification Kits contain two separate bacterial expression vectors encoding N- or C-terminal 6xHN fusion tags (pEcoli-Nterm 6xHN and pEcoli-Cterm 6xHN, respectively), which provide a variety of cloning options (see Figure 2). The kits are available in two formats. The **TALON® Express Bacterial Expression & Purification Kit** (Cat. No. 635639) contains circular vectors with numerous convenient restriction sites for cloning. The **In-Fusion™-Ready TALON® Express Bacterial Expression & Purification Kit** (Cat. No. 635640) contains prelinearized versions of these vectors, utilizing our In-Fusion™ technology to provide easy, low-background directional cloning of PCR products, without the need for restriction enzyme digestion or *in vitro* ligation. See Sections IV and V, respectively, for details regarding these two methods.

Tightly regulated expression

The vectors supplied in these kits contain the hybrid T7 *lac* promoter, as well as a *lacI* gene, which encodes Lac repressor (Dubendorff & Studier, 1991). This combination reduces expression in the absence of inducer, while allowing for rapid inducibility upon addition of IPTG to the bacterial culture (see Figure 3). This reduces background expression levels, since Lac repressor binds to the *lac* operator present adjacent to the T7 promoter.

Easy purification

The provided vectors are designed to incorporate 6xHN tags at the amino- or carboxy-terminus of the protein of interest, allowing for convenient purification using Immobilized Metal ion Affinity Chromatography (IMAC). The kits also include TALON® resin, Clontech's proprietary IMAC resin. TALON resin has a number of advantages over other IMAC resins, including increased specificity.

I. Introduction & Protocol Overview *continued*

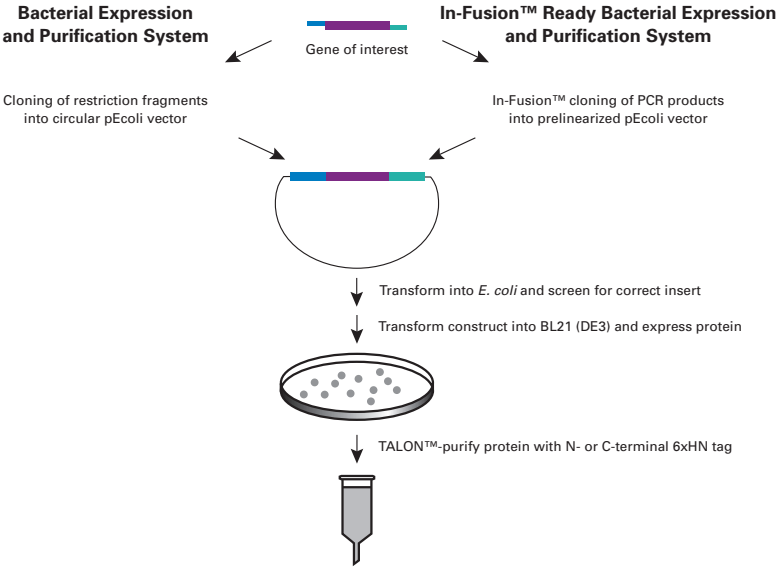

Figure 1. The TALON® Express Bacterial Expression and Purification Kits protocol. A gene of interest is cloned into a pEcoli vector, a pET-based TALON Express Bacterial Expression Vector, to generate an N- or C-terminal-tagged construct. The TALON Express Bacterial Expression and Purification System allows a choice of cloning sites, while the In-Fusion Ready TALON Express Bacterial Expression and Purification System provides the option of easy, precise PCR cloning to yield a 6xHN-tagged construct. After transformation and growth in *E. coli*, the expressed N- or C-terminal-tagged protein is efficiently purified using TALON technology.

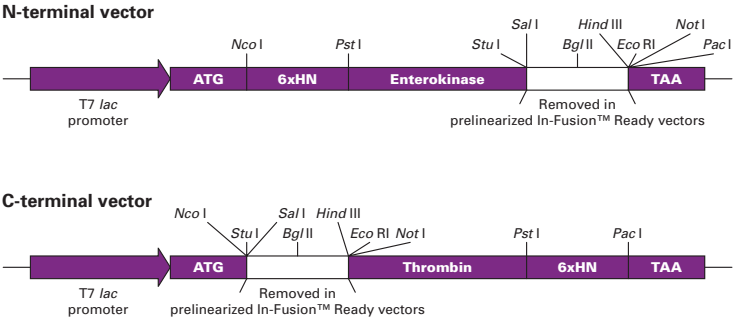

Figure 2. Cloning options using TALON® Express Bacterial Expression Vectors. Features of the multiple cloning sites of our Bacterial Expression Vectors are shown (not to scale). Transcription proceeds from left to right, beginning at ATG and ending at the stop (TAA) codon shown. Convenient restriction sites (indicated) allow inclusion or removal of tags as needed. The unshaded region is removed in our prelinearized vectors using *Sal* I and *Hind* III restriction enzymes.

I. Introduction & Protocol Overview *continued*

TABLE I. TRADITIONAL VS. IN-FUSION™ CLONING USING THE TALON® EXPRESS SYSTEM

Step	Traditional Cloning	In-Fusion Cloning
Steps that differ for the two differerent systems (first part of procedure)		
Prepare vector (Section V)	• Digest with restriction enzymes & dephosphorylate • Gel purify(optional, to reduce background)	• N/A (vector is supplied in linearized form)
Prepare insert (Sections V & VI)	• PCR amplify and/or plasmid prep • Restriction enzyme digestion • Gel purify • Cleanup	• PCR amplify using primers with In-Fusion overhangs • Cleanup
Clone insert into vector (Sections V & VI)	• Combine insert and vector with ligase enzyme and buffer; ligate	• Add vector and insert to In-Fusion Dry-Down mixture; incubate
Steps that are identical for the two different systems (rest of procedure)		
Isolate and verify clones (Section VI)	• Transform into non-expression host strain (eg., Fusion-Blue™ cells) • Colony PCR, expression screening (optional), sequencing	
Transform into expression host (Section VII)	• Transform into BL21 DE3 cells or other DE3 lysogen	
Test and optimize expression (Section VII)	• Grow culture to 0.6–0.8 OD, induce with 1 mM IPTG for 3–6 hr • Check expression with SDS-PAGE and solubility by lysis • Optional: measure time course, temperature; test various strains	
Scale-up (PT1320-1)	• Scale-up culture volume and induce • Lyse cells and prepare extract • Affinity purify cell extract using TALON resin • Cleave affinity tags, remove protease (optional)	

I. Introduction & Protocol Overview *continued*

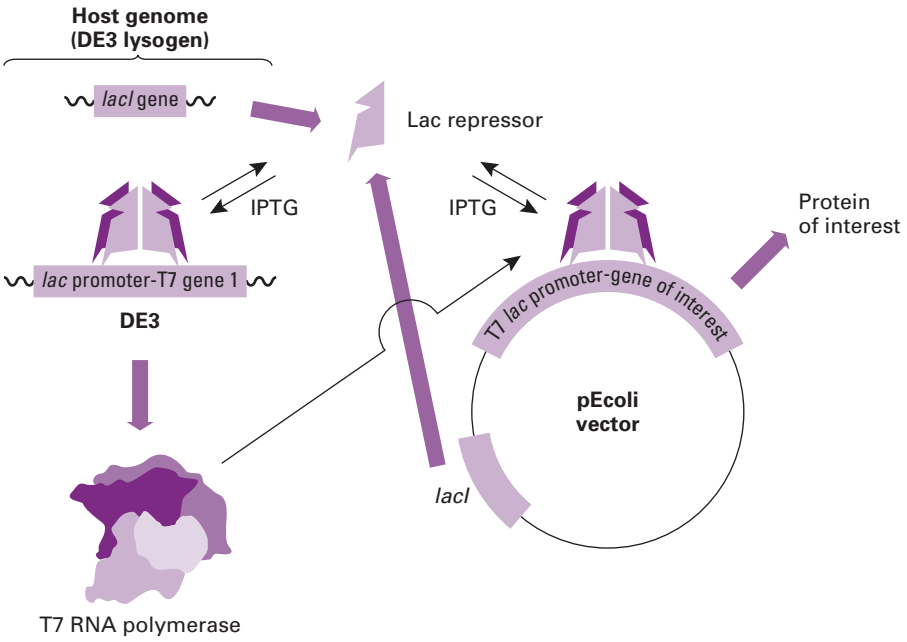

Figure 3. The molecular basis of the pET system for recombinant protein expression in DE3 lysogens. Tight regulation is achieved by the presence of *lac* operator sites in two promoters: the *lac* UV5 promoter that controls expression of T7 RNA polymerase (integrated into the genome of the DE3 lysogen) and the hybrid T7 *lac* promoter that controls expression of the gene of interest. In the absence of inducer (IPTG), Lac repressor (expressed from both genomic and plasmid-derived *lacI* genes) binds tightly to the operator sites to repress transcription. When IPTG is added during induction, Lac repressor releases from the operators, allowing expression of T7 RNA polymerase, which then acts on the newly derepressed T7 *lac* promoter. The thick arrows refer to expression of gene products, while thin arrows indicate molecular interactions.

II. List of Components

- Store columns at room temperature.
- Store buffers and resin at 4°C.
- Store vectors, Test Plasmid, Control Insert, and SOC Medium at –20°C.
- Store Fusion-Blue™ Competent Cells at –70°C.
- Store In-Fusion™ Dry-Down Mix at room temperature, preferably in a desiccator. Once opened, return unused reactions to a sealed bag and/or desiccator.

TALON® Express Bacterial Expression and Purification Kit
(Cat. No. 635639)

- 10 µg pEcoli-Nterm 6xHN Vector (500 ng/µl)
- 10 µg pEcoli-Cterm 6xHN Vector (500 ng/µl)
- 5 µg pEcoli-6xHN-GFPuv Vector (500 ng/µl)
- 10 ml TALON® Metal Affinity Resin
- 160 ml 5X Equilibration/Wash Buffer
  (250 mM sodium phosphate, 1.5 M NaCl, pH 7)
- 160 ml 5X Equilibration Buffer
  (250 mM sodium phosphate, 1.5 M NaCl, pH 8)
- 25 ml 10X Elution Buffer
  (1.5 M imidazole, pH 7)
- 5 2 ml Disposable Columns
- 1 10 ml Disposable Column
- TALON® Express Bacterial Expression and Purification Kit User Manual (PT3856-1)
- TALON® Metal Affinity Resins User Manual (PT1320-1)
- pEcoli-Nterm 6xHN Vector Information Packet (PT3868-5)
- pEcoli-Cterm 6xHN Vector Information Packet (PT3869-5)
- pEcoli-6xHN-GFPuv Vector Information Packet (PT3870-5)

II. List of Components *continued*

In-Fusion™ Ready TALON® Express Bacterial Expression and Purification Kit (Cat. No. 635640)

- 1.5 µg pEcoli-Nterm 6xHN Linear (In-Fusion Ready) Vector (100 ng/µl)
- 1.5 µg pEcoli-Cterm 6xHN Linear (In-Fusion Ready) Vector (100 ng/µl)
- 5 µg pEcoli-6xHN-GFPuv Vector (500 ng/µl)
- 2 ng Test Plasmid (0.2 ng/µl)
- 5 µl 1.1 kb *LacZ*-RK Control Insert (25 ng/µl)
- 10 Fusion-Blue™ Competent Cells (50 µl/tube)
- 2 SOC Medium (2 ml/tube)
- 8 rxn In-Fusion™ Dry-Down Mix (1 x 8-well strip)
- 10 ml TALON® Metal Affinity Resin
- 160 ml 5X Equilibration/Wash Buffer (250 mM sodium phosphate, 1.5 M NaCl, pH 7)
- 160 ml 5X Equilibration Buffer (250 mM sodium phosphate, 1.5 M NaCl, pH 8)
- 25 ml 10X Elution Buffer (1.5 M imidazole, pH 7)
- 5 2 ml Disposable Columns
- 1 10 ml Disposable Column
- TALON® Express Bacterial Expression and Purification Kit User Manual (PT3856-1)
- TALON® Metal Affinity Resins User Manual (PT1320-1)
- pEcoli-Nterm 6xHN Linear (In-Fusion™ Ready) Vector Information Packet (PT3871-5)
- pEcoli-Cterm 6xHN Linear (In-Fusion™ Ready) Vector Information Packet (PT3872-5)
- pEcoli-6xHN-GFPuv Vector Information Packet (PT3870-5)

III. Additional Materials Required

The following materials are required but not supplied:

- **Advantage® HF 2 Polymerase Mix** (Cat. Nos. 639123 & 639124)
- **NucleoSpin® Extract II Kit** (Cat. No. 636972)
- **NucleoTrap Gel Extraction Kit** (Cat. No. 636018) [Optional]
- **TALON® xTractor Buffer Kit** (Cat. No. 635623 & 635625)
  (kit includes lysozyme as well as buffer)
- **Complete Protease Inhibitor Cocktail Tablets** [Roche Applied Science, Cat. Nos. 1 836 153 (for 10 ml) & 1 697 498 (for 50 ml)]
- **5X SDS loading buffer**
  15% β-Mercaptoethanol
  15% SDS
  50% Glycerol
  1.5% Bromophenol Blue
- **BL21 (DE3) competent bacteria** (EMD Biosciences, Inc.)
- **Ampicillin** (100 mg/ml stock)
- **LB (Luria-Bertani) medium** (pH 7.0)

		for 1 L
1.0%	Bacto-tryptone	10 g
0.5%	Yeast extract	5 g
1.0%	NaCl	10 g

  Dissolve ingredients in 950 ml of deionized H_2O. Adjust the pH to 7.0 with 5 M NaOH and bring the volume up to 1 L. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Store at room temperature or at 4°C.

- **LB/antibiotic plates**

  Prepare LB medium as above, but add 15 g/L of agar before autoclaving. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Let cool to ~55°C, add antibiotic (e.g., 100 µg/ml of ampicillin), and pour into 10 cm plates. After the plates harden, then invert and store at 4°C. For LB/X-Gal/IPTG plates, spread 40 µl each of X-Gal and IPTG stock solutions on an LB plate.

- **X-Gal stock solution** (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 40 mg/ml in dimethylformamide)

  Dissolve 400 mg of X-Gal in 10 ml of dimethylformamide. Protect from light by storing in a brown bottle at –20°C.

- **IPTG stock solution** (isopropyl-β-D-thiogalactoside; 100 mM)

  Dissolve 238 mg of IPTG in 10 ml of deionized H2O. Filter-sterilize and store in 1 ml aliquots at –20°C.

IV. General Considerations

To clone your gene of interest, you may use either the **TALON® Express Bacterial Expression & Purification Kit** (Cat. No. 635639), which contains circular vectors for traditional restriction enzyme-based cloning, or the **In-Fusion™ Ready TALON® Express Bacterial Expression & Purification Kit** (Cat. No. 635640), which contains prelinearized vectors designed to be used with Clontech's In-Fusion™ technology.

Using traditional cloning methods (i.e., ligation-based cloning of compatible DNA fragments) allows the choice of several cloning sites (Figure 2), provided that these sites are not found internally in the DNA fragment to be cloned. Traditional cloning may be more convenient If the fragment to be cloned contains restriction sites in frame with the gene of interest.

Otherwise, In-Fusion cloning provides several advantages over restriction enzyme-based cloning:

- In-Fusion cloning technology allows for directional cloning of PCR products into In-Fusion Ready vectors without the need for additional restriction digests, ligation, or blunt end polishing. This is more convenient than restriction enzyme-based cloning, and allows the use of restriction sites that are present within the fragment to be cloned.
- Since the vectors are provided in prelinearized form (using *Sal* I and *Hind* III), there is no need to digest the vector prior to cloning. In-Fusion cloning is based on homology between the ends of the linearized vector and the PCR product— this homology is introduced into the PCR product via the use of PCR primers with 5' extensions.
- The pEcoli-Nterm 6xHN and pEcoli-Cterm 6xHN vectors have been designed so that the sequences 5' to the *Sal* I site and 3' to the *Hind* III site are the same in both vectors. This allows cloning of the same PCR product into both vectors in parallel, which is convenient for comparing expression and purification of your protein when an 6xHN tag is attached to either the amino or the carboxy terminus, respectively. Furthermore, these PCR products are compatible with all of our In-Fusion Ready prelinearized fluorescent protein vectors, allowing you to easily switch to a different expression system, depending on your needs.
- Everything you need to know about cloning into our In-Fusion Ready TALON Express Bacterial Expression Vectors can be found in this manual. For more information on In-Fusion technology, including instructions on how to use In-Fusion technology with any vector, download our In-Fusion Dry-Down PCR Cloning Kit User Manual (PT3754-1), at

  www.clontech.com/clontech/techinfo/manuals/

V. Traditional Cloning Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Traditional cloning consists of many steps. These include primer design, PCR amplification, and cleanup of the PCR insert; or cleavage from an existing vector and insert cleanup. The vector must also be cleaved and cleaned up. This is followed by T4 DNA ligation, transformation, and screening of inserts.

A. Cloning Site Selection and PCR Primer Design

Use the following criteria to select restriction sites for cloning and to design PCR primers for generating the DNA insert:

- Choose appropriate restriction sites based on the absence of these sites within the DNA insert to be cloned.
- These sites should be incorporated into PCR primers which are then used to PCR-amplify the sequence to be cloned.
- These primers should be carefully designed to ensure that the reading frame of your gene aligns with the ATG start codon which forms part of the *Nco* I site at the 5' end of the MCS.
- Make certain to design your 5' primer **without** the ATG start codon of the wild type protein.
- Sufficient flanking sequence should be incorporated to ensure efficient digestion of the PCR product using the appropriate restriction enzymes. We recommend six nucleotides 5' to the RE recognition sequence; consult the New England Biolabs catalog appendix "Cleavage Close to the End of DNA Fragments (linearized vector)" for more information:

  www.neb.com/nebecomm/tech_reference/restriction_enzymes/cleavage_linearized_vector.asp

  Notes:

  - The MCS is designed with overlapping *Pac* I sites at the 3' end. This ensures that all three reading frames contain a stop codon. If the *Pac* I site is used for cloning, only one of the sites in the vector will be cut. Thus, be sure that your intended stop codon is found in the first *Pac* I site. That way, the stop codon will be in frame regardless of which *Pac* I site in the vector is digested.
  - The *Xba* I site is 3' to the *Pac* I sites, and is therefore not followed by stop codons. If you use the *Xba* I site for cloning, be sure that your insert contains its own stop codon.

V. Traditional Cloning Protocol *continued*

B. Generating a Gene-Specific Expression Vector

Generate your recombinant expression construct using standard molecular biology techniques, as described below. For more detailed information, see Molecular Cloning: A Laboratory Manual (Sambrook & Russell, 2001) or Current Protocols in Molecular Biology (Ausubel. *et al*., 2001).

1. Digest the pEcoli vector with the restriction enzyme(s) appropriate for your expression application, treat with phosphatase (if desired), and purify.

   Notes:

   - The ends of the fragment to be cloned must be compatible with one or more restriction sites present in the cloning vector. This can be accomplished by PCR amplifying the fragment using primers that contain suitable restriction sites.
   - If cloning with the 6xHN affinity tag included in the vector, ensure that the correct reading frame is maintained (see the MCS of the appropriate vector for the alignment of restriction sites with the open reading frame that begins with the initiation codon (ATG) within the *Nco* I site).

2. Purify the insert using any standard method.
3. Ligate the digested vector and the gene fragment.
4. Transform chemically competent or electrocompetent bacterial cells (such as our Fusion-Blue™ Competent Cells; Cat. No. 636700) with a sample of the ligation mixture and plate on LB agar plates containing 100 µg/ml ampicillin.
6. Identify the desired recombinant plasmid by colony PCR, restriction analysis of miniprepped DNA, and/or sequencing of miniprepped DNA.
7. Transform into BL21 DE3 or other strain containing DE3 for expression analysis (see Section VII).

VI. In-Fusion Cloning Protocol

In-Fusion cloning consists of several steps. These include primer design, PCR amplification of the insert, cleanup of the PCR insert, the In-Fusion cloning reaction, bacterial transformation, and screening of transformants.

A. Primer Design

In-Fusion cloning requires that the PCR insert contains 15 bases of flanking sequence that match the ends of the vector. This is accomplished by adding these sequences at the 5' ends of the sense and antisense primers.

1. General design guidelines
   - **Sense primer:** 5'-TAAGGCCTCT**GTCGAC** followed by sufficient sequence matching the start (or 5' end) of the gene of interest to ensure efficient annealing for PCR amplification. Make sure that your gene specific sequence does not contain the ATG start codon of your wild type protein.
   - **Antisense primer:** 5'-CAGAATTCGC**AAGCTT** followed by sufficient sequence matching the end (or 3' end) of the gene of interest to ensure efficient annealing for PCR amplification.
2. Ensuring an in-frame sequence
   - The *Sal* I and *Hind* III sites (**GTCGAC** in the sense primer and **AAGCTT** in the antisense primer) each comprise two codons of the translational reading frame. To ensure that your sequence is in frame with the ATG start codon and the N-terminal or C-terminal tags, design your primers such that complete codons are adjacent to these sites, as shown in Figure 8 for pEcoli-6xHN-GFPuv Vector.
   - PCR products generated with these primers will clone in frame into all In-Fusion Ready prelinearized fluorescent protein vectors. Since the vectors contain ATG start codons in optimized positions, it is not recommended to include the ATG of the gene to be cloned. Any translational initiation starting with this ATG will result in proteins lacking N-terminal tags.
3. Primer purification requirements

   We generally use desalted oligo primers in PCR reactions. However, oligo quality can depend on the vendor and varies from lot to lot. If your oligo supply is particularly poor (i.e., has a lot of premature termination products), or your PCR primer is longer than 45 nucleotides, you may need to use SDS-PAGE purified oligos, but in general we find that this is unecessary.

B. PCR Amplification and Cleanup

1. Generation of PCR Templates
   - In general, 10–100 ng of DNA is sufficient for use as a PCR template. However, if you are amplifying a pool of cDNA, the amount of template DNA depends on the relative abundance of the target message in your mRNA population.

VI. In-Fusion Cloning Protocol *continued*

- For best results, we recommend using our Advantage® HF 2 Polymerase Mix (Cat. Nos. 639123 & 639124), which offers high-fidelity, efficient amplification of long gene segments (>1 kb), and an automatic hot start that reduces nonspecific products. Hot start PCR is commonly used to enhance the specificity and sensitivity of PCR amplification (D'Aquila *et al.*, 1991; Chou *et al.*, 1992; Faloona *et al.*, 1990). We offer the TaqStart™ Antibody (Cat. No. 639250) for automatic hot start PCR with other *Taq*-based polymerase mixes. Since primers and primer dimers are inhibitory to the In-Fusion PCR cloning reaction, we recommend using hot start PCR with a touchdown protocol to increase the specificity of the resulting PCR products.
- **Important:** If your PCR template is a plasmid DNA, then cut any contaminating linear vector by incubating the 50–100 µl PCR reaction mix with 1 µl of *Dpn* I for 30 min at 37°C before purifying your PCR products.

2. Analysis of PCR Products
   - When cycling is complete, analyze your PCR product by electrophoresis on an agarose/EtBr gel to confirm that you have obtained a single DNA fragment and to estimate the concentration of your PCR product. Quantify the amount of DNA by measuring against a known standard or molecular weight marker ladder run on the same gel. The *Lac*Z-RK Control Insert or the linear vectors, provided in the kit, are useful for this purpose.
   - The percentage of agarose and the DNA size markers you choose will depend on the expected range of insert sizes. These are general guidelines:

Expected size	% agarose	DNA size markers
0.3–1.5 kb	1.5	fX174/*Hae* III
0.5–10 kb	1.2	1 kb DNA ladder
>5 kb	0.8	l/*Hin*d III

3. Cleanup or Purification of PCR Products
   - PCR products must be cleaned up or purified for successful In-Fusion cloning. The method of purification required depends on your gel electrophoresis results. If you observe only a single, clear band on the gel corresponding to your product, then removal of unincorporated dNTPs through a simple PCR cleanup is usually sufficient. We recommend the NucleoSpin Extract II Kit (Cat. No. 636972) using the protocol for isolation from PCR.
   - If, however, multiple bands are observed, indicating the presence of nonspecific contaminants, we recommend that you gel purify your fragment of interest. We have found that either electroelution

VI. In-Fusion Cloning Protocol *continued*

or silica-based DNA purification systems, such as the NucleoTrap Gel Extraction Kit (Cat. No. 636018) or the NucleoSpin® Extract II Kit (Cat. No. 636972) work well.

- During purification, be careful of nuclease contamination and avoid exposing the DNA to UV light for long periods of time. All solutions that come in contact with the gel and fragment should be free of nucleases. Avoid communal EtBr baths and use only high-quality agarose.

TABLE II. RECOMMENDED IN-FUSION™ REACTIONS AND CONTROL REACTIONS

Reaction No.	1[a]	2	3	4
Rxn Component	Cloning Rxn (your PCR insert)	Positive Control (*lac*Z-RK insert)	Negative Control (vector alone)	Negative Control (insert alone)
PCR insert	50–100 ng[b]	50 ng (2 µl)	—	same as Rxn No. 1
Linearized vector	150 ng (1.5 µl)	150 ng (1.5 µl)	150 ng (1.5 µl)	—
Deionized water	to 10 µl	to 10 µl	to 10 µl	to 10 µl
Add to In-Fusion Dry-Down pellet	Yes	Yes	Yes	No[a]

[a] Any background from "Insert alone" is indicative of incomplete DpnI digestion of a PCR template carrying ampicillin. Incubation with In-Fusion enzyme will not alter this background.
[b] See Section D for recommended insert concentration.

C. Control Reactions

If you are performing In-Fusion PCR cloning for the first time, we strongly recommend that you carry out the positive and negative control reactions (see Table II) in parallel with your cloning reaction. Performing the control reactions will verify that your system is working properly.

D. Cloning Procedure

In general, maximum cloning efficiency is achieved when using a 2:1 molar ratio of insert:vector. Typically 150 ng of a 6 kb linearized cloning vector plus 50 ng of a 1 kb PCR fragment is found to clone well in a 10 µl In-Fusion reaction (see Table II). Adjust the amount of your input DNA if the size of your vector or PCR fragment are different from above.

1. Mix your PCR fragment & vector together at a 2:1 molar ratio in 10 µl of deionized H_2O.
2. Set up the In-Fusion cloning reactions:
   a. Cut one tube off the strip, and peel back the aluminum seal.
   b. Add the 10 µl of vector + insert DNA (from Step 1) and mix well by pipetting up and down.
3. Incubate reactions at 42°C for 30 min, then transfer tubes to ice.
   Note: In-Fusion reactions can also be performed at room temperature; however we have observed improved efficiency at 42°C for vectors larger than 5 kb.
4. Dilute each reaction with 40 µl TE buffer.

VI. In-Fusion Cloning Protocol *continued*

5. Proceed with transformation (Section E). If you cannot transform cells immediately, store cloning reactions at –20°C until you are ready.

E. Transformation

In addition to the cloning reactions listed in Table II, we recommend that you perform a positive control transformation. Clontech's Fusion-Blue™ Competent Cells (Cat. No. 636700) should yield a transformation efficiency >1.5 x 10^8 cfu/µg using the supplied test plasmid. We recommend using *E. coli* cells that have a competency of at least 1x 10^8 cfu/µg DNA.

1. For each transformation, thaw one vial of frozen Fusion Blue Competent Cells on ice. Tap tube gently to ensure that the cells are suspended. For the control transformation, thaw the test plasmid.
2. Add 2.5 µl of each diluted reaction mixture to a separate vial of cells. Do not add more than 5 µl of diluted reaction to 50 µl of competent cells. For the control transformation, add 1 µl (0.2 ng) of the test plasmid to a vial of cells.
3. Mix gently to ensure even distribution of the DNA. Incubate on ice for 30 min.
4. Heat shock the cells in a water bath at 42°C for 45 sec, and then place the tubes on ice for at 1–2 min.
5. Add 450 µl SOC medium to the cells and then incubate at 37°C for 60 min while shaking at ~250 rpm.
6. Plate transformations as follows:
   a. In-Fusion reactions
      i. Take 1/20–1/10 of the cells (25–50 µl) from each transformation, bring the volume to 100 µl with SOC medium, and then spread on separate LB/amp plates (ampicillin concentration is 100 µg/ml).
      Note: If desired, plate Reaction No. 4 on LB/Amp/IPTG/X-Gal plates.
      ii. Centrifuge 1 min at 6,000 x g to pellet remaining cells. Re-suspend in ~100 µl medium (this is most easily accomplished by first removing all but ~100 µl from the tubes), and plate on LB/amp plates.
   b. Control transformations
      Dilute transformations 10X and 100X by adding 110 µl and 10 µl of each transformation, respectively, to separate aliquots of 990 µl SOC or LB. Then mix well and plate 100 µl of each dilution on LB/amp plates labeled “10X” and “100X”.
7. Incubate all plates at 37°C overnight. To determine transformation efficiency (i.e., the number of colonies per µg of DNA), multiply the number of colonies on the 100X dilution plate by 2.5 x 10^5, or the 10X dilution plate by 2.5 x 10^4. The transformation efficiency should be at least 1 x 10^8, which corresponds to 80 colonies on the 100X plate and 800 colonies on the 10X plate.

VI. In-Fusion Cloning Protocol *continued*

8. Expected results

   The transformation plates from Reactions No. 1 (vector + your PCR insert) and 2 (vector + control insert) should contain at least 5 times as many colonies as the plates from Reaction No. 3 (vector alone). The plates from Reaction No. 4 (insert alone) should contain very few colonies and then only if the PCR template is a plasmid conferring ampicillin resistance. The presence of many colonies suggests incomplete *Dpn* I digestion following PCR. In such cases it is recommended that the process be repeated beginning with the PCR reaction.

F. Screening colonies for the insert

Colonies can be screened for the correct insert by colony PCR, or DNA may be miniprepped and screened by restriction digestion or sequencing. Because In-Fusion cloning involves PCR, it is recommended that one or more clones be confirmed by sequencing.

The following primers are recommended for sequencing or colony PCR (see provided Vector Information Packets listed in Section II):

- T7-Up1 sequencing primer:
  5'-CGGCGTAGAGGATCGAG-3'
- T7 terminator sequencing primer:
  5'-CTAGTTATTGCTCAGCGG-3'

VII. Protein Expression Protocol

A. General Considerations

Once the desired expression plasmid has been obtained, you may induce expression of your protein using IPTG. First, the plasmid must be transformed into an *E. coli* strain that contains the T7 polymerase gene under the control of the *lac* repressor, such as BL21 DE3. BL21 cells are Lon and OmpA protease-deficient, and are thus ideal hosts for overexpression of ectopic proteins. BL21 DE3 cells are a derivative of BL21 that contain an integration of the T7 polymerase-expressing lysogen DE3, making them compatible with the T7-based pET system.

- Competent BL21 (DE3) cells are available from EMD Biosciences, Inc. (see Section III). These should be transformed according to the manufacturer's instructions.
- It is recommended to use freshly transformed bacteria or frozen glycerol stocks for expression experiments, as continued culture or long periods of storage on plates can result in loss of expression.
- The following protocols are provided for the analysis of protein expression, including protein solubility. This small-scale approach is also useful for optimizing expression conditions. The expression protocol can be scaled up for production, and IMAC purification can be performed using the protocols in the TALON Metal Affinity Resins User Manual PT1320-1 (included).

B. Expression Protocol (Small Scale)

1. Pick colonies to innoculate small (2 to 4 ml) LB/amp cultures and shake overnight at 37°C (ampicillin concentration is 100 µg/ml).

   In the morning, innoculate a 4 to 50 ml culture by diluting the overnight culture 1/20 to 1/50 with LB amp.
2. Shake until an OD of 0.6–0.8 is reached, set aside a small volume of culture (~1 ml) on ice to serve as an uninduced control, and then add IPTG to a concentration of 1mM. Continue shaking at 37°C for 4–5 hr.
3. Centrifuge samples at 1,000–3,000 x g for 15 min at 4°C, remove supernatant and freeze pellets at –80°C for further analysis.

C. Analysis of Expression

You have the option to analyze total protein expression, or proceed directly to analyzing solubility (see Section D). The former approach is useful when analyzing a number of samples. The latter approach will determine whether the protein is soluble, which is essential when planning the purification protocol since some eukaryotic proteins are insoluble when expressed in bacteria and form inclusion bodies. The trade-off of insolubility is that these proteins tend to be expressed at high levels, and isolation of inclusion bodies is an effective initial purification step.

VII. Protein Expression Protocol *continued*

Total expression can be analyzed by SDS-PAGE as follows:

1. Resuspend pellets in 1/10th of the original culture volume using PBS, add an equal volume of 2X SDS Loading Buffer (see Section III), and mix well (it is ok to mix samples on a vortex mixer, but avoid forming bubbles if you intend to sonicate the samples).
2. The samples can then be sonicated for 30 sec, or drawn through a 27 gauge needle 4 or 5 times. This will reduce the viscosity of the sample due to genomic DNA and thus facilitate gel loading. An alternative is to centrifuge the sample for 5 min just prior to loading the gel and use the supernatant, taking care to avoid the pellet when pipetting.
3. Heat the sample to 95°C for 3 to 5 minutes, centrifuge briefly and proceed to load the gel. Samples may be stored at -20°C.

D. Analysis of Solubility

There are numerous successful protocols for improving solubility and refolding proteins from inclusion bodies. In the latter case, the protein must be solubilized using denaturants prior to purification. IMAC purification using TALON resin works under both native and denaturing conditions.This protocol will allow you to separate soluble and insoluble (inclusion body) fractions and then analyze these by SDS-PAGE.

1. Resuspend the cell pellets in TALON xTractor Buffer:
   a. Add 20 µl TALON xTractor Buffer (see Section III) per mg of cell pellet or 40 µl per ml of the original culture volume.
   b. Incubate at room temperature for 20–30 min (the incubation with TALON xTractor buffer can be carried out on ice for 45–60 min).

   Note: Add DNAse to TALON xTractor Buffer if the resulting sample is very viscous.
2. Alternatively, resuspend the cell pellets in 1X Equilibration/Wash Buffer:
   a. Resuspend the cell pellets in a volume of the provided 1X Equilibration/Wash Buffer equivalent to 1/10th the original culture volume (chilled to 4°C).
   b. Add lysozyme to a concentration of 0.75 mg/ml. To reduce the chance of introducing proteases, use the highest purity lysozyme available.
   c. Sonicate your sample 3 x 10 sec, with a 30 sec pause on ice between each burst.
3. Centrifuge the cell extract at 10,000–12,000 x g for 20 min at 4°C to pellet insoluble material.
4. Carefully transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.

VII. Protein Expression Protocol *continued*

5. Reserve a portion for SDS/PAGE analysis. The remainder may be kept for a trial purification using TALON resin. Keep the reminder on ice until used for purification (carry out the purification within 2–4 hr after the extraction).
6. Resuspend the pellet in a volume of 1X Equilibration/Wash Buffer equivalent to the volume used in Step 1. Add an equal volume of 2X SDS sample buffer.
7. The resuspended pellet can then be sonicated for 30 sec, or drawn through a 27 gauge needle 4 or 5 times. This will reduce the viscosity of the sample due to genomic DNA, and thus facilitate gel loading. An alternative is to centrifuge the sample for 5 min just prior to loading the gel, and take care to avoid the pellet when pipetting.
8. Heat the samples corresponding to both the soluble and insoluble fractions at 95°C for 3–5 min and centrifuge briefly.
9. Analyze samples by SDS-PAGE. Add to this an equivalent volume of 2X SDS Sample Buffer. Typically, a sample equivalent to 25–50 µl of culture volume will produce clean bands on a gel. This corresponds to 5–10 µl of sample when processed according to the above instructions
10. If you wish to identify the target protein band, use our Universal HIS Western Blot Kit (Cat. No. 635633) after transferring the bands to a PVDF membrane.

E. TALON® Purification

For general considerations and purification protocols using TALON resin, consult the provided TALON Metal Affinity Resins User Manual (PT1320-1). Follow the instructions provided for the TALON Purification Kit (Cat. No. 635515) in **Sections IV & VIII.B of PT1320-1**.

VIII. Troubleshooting Guide for In-Fusion Cloning

If you do not obtain the expected results, use the following guide to troubleshoot your experiment. To confirm that your kit is working properly, perform the control reactions (Section V.C).

A. No or few colonies obtained from transformation

Problems with transformation

Inhibitory contaminants in PCR product	Repeat PCR amplification and purify product using a different method of purification. Alternatively, perform phenol:chloroform extraction on your original PCR product, followed by ethanol precipitation.
Bacteria were not competent	**Check transformation efficiency.** You should obtain **>1 x 10^8 cfu/µg**; otherwise use fresh competent cells.
Transformed with too much reaction mixture	**Do not add >5 µl of diluted In-Fusion reaction** (1 µl of undiluted reaction) **to 50 µl of competent cells**. More is not better. Using too much of the reaction mixture inhibits the transformation. For example, 2.5 to 5 µl of a diluted In-Fusion reaction in 50 µl of cells typically yields hundreds of colonies, while 10 µl of the same reaction will yield less than 100 colonies. Since it can be difficult to to pipette 1 µl accurately (e.g., if you are using "yellow tips" with a P20 pipettor), dilute your In-Fusion reaction with TE buffer (pH 8) before performing the transformation—especially if you wish to use a small volume of competent cells (e.g., HTP cloning). See Section VI.E for details.

Problems with cloning reaction

Cloning reaction failed	Check the reaction for a shift in vector size by running half of the reaction on a 1% agarose gel. In extremely rare cases, the In-Fusion reaction may fail to clone certain sequences effectively. The In-Fusion cloning reaction depends on sequence homology, so sequences containing extensive or multiple repeats may not clone efficiently. In these cases, an alternative cloning strategy might be needed.
Low DNA concentration in reaction	Either the amount of vector or the amount of PCR fragment was too low to obtain a satisfactory reaction product (Figure 4). Alternatively, the 2:1 molar ratio of PCR fragment to linear vector used in the In-Fusion protocol may not have been optimal.

VIII. Troubleshooting Guide for In-Fusion Cloning *continued*

Primer sequences incorrect	Check primer sequences to ensure that they provide 15 bases of homology with the region flanking the vector cloning site (see Section IV) if using In-Fusion cloning.
Low cloning efficiency	Check the concentration of your PCR fragment. Do not use less than 25 ng or more than 100 ng of your fragment in the In-Fusion cloning reaction; the cloning efficiency may be reduced if you exceed these parameters.

B. Clones contain incorrect insert

Contamination of PCR product with nonspecific sequences.	If your PCR product is not a single distinct band, then it may be necessary to gel purify the PCR product to ensure cloning of the correct insert. See Section VI.B.3 for more information.

IX. Troubleshooting Guide for Protein Expression

A. Transformation of BL21 DE3 cells yields no colonies

Protein is toxic to cells	Try BL21 DE3 pLysS or pLysE cells. These strains contain plasmids which express T7 lysozyme, an inhibitor of T7 polymerase, thus raising the threshold of T7 polymerase required for expression of the gene of interest.

B. Protein is not present in cells

Incorrect cells used for transformation	Use BL21 DE3 cells or other cells which express T7 polymerase under a *lac*-regulated promoter
Clones contain incorrect insert	Pick another transformant.
Protein is toxic to cells	Try BL21 DE3 pLysS or pLysE cells. These strains contain plasmids which express T7 lysozyme, an inhibitor of T7 polymerase, thus raising the threshold of T7 polymerase required for expression of the gene of interest.
Protein may require longer induction time	Increase culture incubation time. The incubation may be performed overnight.
Protein is degraded	Try adding protease inhibitors (Roche Complete Protease Inhibitor Cocktail Tablets, Cat. No. 1 836 153 (for 10 ml) or Cat. No. 1 697 498 (for 50 ml)

C. Protein is insoluble

Confirm presence of protein by lysing cell pellet using SDS-PAGE loading buffer and rescue solubility by altering expression conditions or expressing the protein within inclusion bodies.

- **Alter expression conditions to improve solubility by promoting correct protein folding**
  1. Reduce the induction temperature to slow expression and allow time for proper folding. Try a few temperatures between 15°C and 30°C.
  2. Refold protein in the presence of chaperones by transforming plasmids that express a variety of chaperones [eg., Takara Chaperone Plasmid Set (Cat. No. 3340)], or by expressing your protein in derivatives of BL21 DE3 cells that express chaperones.

- **Express the protein in insoluble form within inclusion bodies, then denature and refold**

  Since insoluble proteins will typically be expressed at high levels, isolation of inclusion bodies containing your protein of interest with one of the following two methods is an excellent purification step:

IX.Troubleshooting Guide for Protein Expression *cont...*

1. Denature the proteins within the inclusion bodies in a solution of 8M Urea or 6M Guanidine Hydrochloride, then perform a gradual dilution of the denatured protein solution using stepwise dialysis. Since IMAC purification works under denaturing conditions, one has the option to purify either before or following refolding.
2. Use a commercially available refolding kit (eg. Takara Refolding CA Kits, Cat. Nos. 7350 & 7351).

X. References

Ausubel, F. M. , Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., Eds. (2001) *Current Protocols in Molecular Biology* (John Wiley and Sons, Inc., NY).

Chou, Q., Russell, M., Birch, D., Raymond, J. & Block, W. (1992) Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res.* **20**:1717–1723.

Bacterial Expression and Purification Kits (October 2005) *Clontechniques* **XX**(2):12-13.

D'aquila, R. T., Bechtel, L. J., Videler, J. A., Eron, J. J., Gorczyca, P. & Kaplan, J. C. (1991) Maximizing sensitivity and specificity of PCR by preamplification heating. *Nucleic Acids Res.* **19**:3749.

Dubendorff, J. W. and Studier, F. W. (1991) Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with *lac* repressor. *J. Mol. Biol.* **219**(1):45-59.

Faloona, F., Weiss, S., Ferre, F. & Mullis, K. (1990) Direct detection of HIV sequences in blood: high-gain polymerase chain reaction. 6th Int'l Conf. AIDS, San Francisco, CA; Abstr. Cat. No. 1019.

McMurry J.L. and Macnab R.M. (January 2004) *Clontechniques* **XIX**(1):16-17.

Moffatt, B. A. and Studier, F. W. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**(1):113-130.

Rosenberg, A. H. and Studier, F. W. (1987) T7 RNA polymerase can direct expression of influenza virus cap-binding protein (PB2) in Escherichia coli. *Gene* **59**(2-3):191-200.

Sambrook, J. & Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**:60-89.

TALON® Metal Affinity Resin (January 2001) *Clontechniques* **XIX**(1):16-17.

XI. Related Products

For a complete listing of all Clontech products,please visit **www.clontech.com**

Product	Cat. No.
• Advantage® HF 2 Polymerase Mix	639123 639124
• In-Fusion Dry-Down PCR Cloning Kit	639602 639604 639605 639606
• Fusion-Blue™ Competent Cells	636700 636758
• NucleoSpin® Extract II Kit	636972
• NucleoTrap Gel Extraction Kit	636018
• TALON® xTractor Buffer Kit	635623 635625
• TALON® Purification Kit	635515
• TALON® Superflow Metal Affinity Resin	635506 635507
• TALON® Single Step Protein Purification Columns	
5 ml	635628 635631
20 ml	635632
• Universal HIS Western Blot Kit	635633
• 6xHN Polyclonal Antibody	631213

Appendix: Vector Information

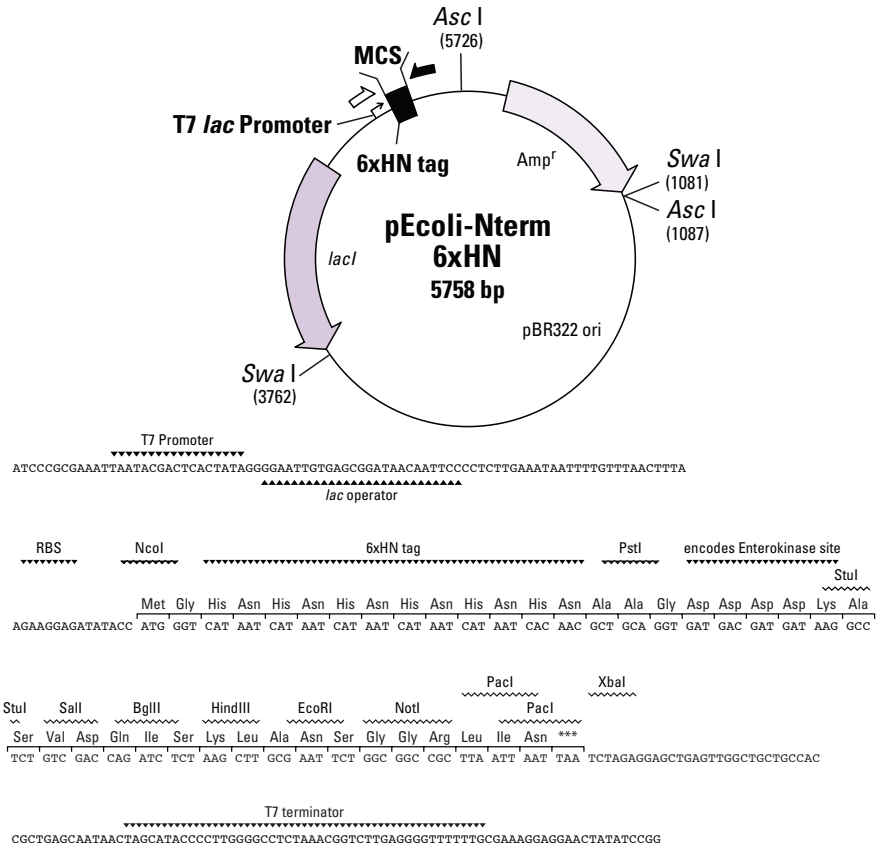

Figure 4. pEcoli-Nterm 6xHN Vector Map and MCS. pEcoli-Nterm 6xHN is a highly inducible bacterial expression vector based on the pET system (Dubendorf & Studier, 1991; Studier & Moffatt, 1986; Rosenberg *et al.*, 1987, Studier *et al.*, 1990). This vector, a derivative of pET11 (Dubendorf and Studier, 1991), combines the strongly induced T7 promoter with an operator for the *lac* repressor to produce tightly regulated expression. The multiple cloning site (MCS) contains coding sequences for an N-terminal 6xHN immobilized metal affinity chromatography (IMAC) purification and detection tag, $(His\text{-}Asn)_6$, suitable for protein purification using TALON resin, as well as coding sequences for an enterokinase cleavage site $(Asp)_4$-Lys. The unshaded and black arrows on the left and right sides of the MCS, respectively, represent the positions of the recommended forward (T7-Up1) and reverse (T7 terminator) sequencing primers (see Section VI.F for sequences). The vector expresses Lac repressor from the *lacI* gene, which represses expression from the T7 promoter in the absence of the inducer IPTG. pEcoli-Nterm 6xHN also contains an ampicillin resistance marker and a pBR322 origin of replication, which maintains the plasmid at a low-copy-number to further reduce expression in the uninduced state. The ampicillin resistance marker (Amp^r) can be replaced using the flanking *Asc* I sites, while the pBR322 origin of replication can be replaced using the flanking *Swa* I sites. Any of the restriction sites shown in the MCS can be used for cloning your gene of interest; you must incorporate a stop codon in your PCR product if *Pac* I or *Xba* I sites are used; since use of these sites may eliminate the existing stop codon (marked ***). Several MCS restriction sites are present in the same reading frame in both pEcoli-Nterm 6xHN and pEcoli-Cterm 6xHN vectors, namely *Stu* I, *Sal* I, *Bgl* II, *Hind* III, *Eco* RI and *Not* I. If these sites are used for cloning, the same insert can be used for both vectors. All restriction sites shown in the MCS, except for the *Pac* I sites, are unique. The pEcoli-Nterm 6xHN Linear (In-Fusion Ready) Vector (Figure 6) included in the TALON Express Bacterial Expression and Purification Kit is designed for convenient In-Fusion cloning using the *Sal* I and *Hind* III sites.

Appendix: Vector Information *continued*

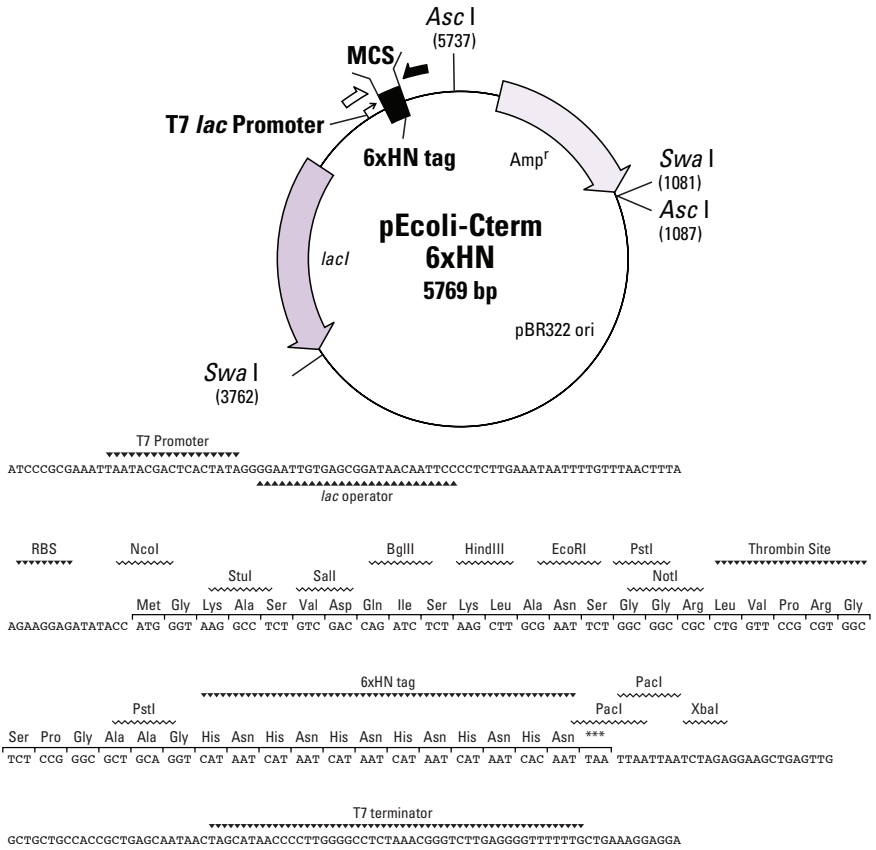

Figure 5. pEcoli-Cterm 6xHN Vector Map and MCS. pEcoli-Cterm 6xHN is a highly inducible bacterial expression vector based on the pET system (Dubendorf & Studier, 1991; Studier & Moffatt, 1986; Rosenberg *et al.*, 1987, Studier *et al.*, 1990). This vector, a derivative of pET11 (Dubendorf and Studier, 1991), combines the strongly induced T7 promoter with an operator for the *lac* repressor to produce tightly regulated expression. The multiple cloning site (MCS) contains coding sequences for a C-terminal 6xHN immobilized metal affinity chromatography (IMAC) purification and detection tag, $(His\text{-}Asn)_6$, suitable for protein purification using TALON resin, as well as coding sequences for a thrombin (LVPRGS) cleavage site. The unshaded and black arrows on the left and right sides of the MCS, respectively, represent the positions of the recommended forward (T7-Up1) and reverse (T7 terminator) sequencing primers (see Section VI.F for sequences). The vector expresses Lac repressor from the *lacI* gene, which represses expression from the T7 promoter in the absence of the inducer IPTG. pEcoli-Cterm 6xHN also contains an ampicillin resistance marker and a pBR322 origin of replication, which maintains the plasmid at a low-copy-number to further reduce expression in the uninduced state. The ampicillin resistance marker (Amp^r) can be replaced using the flanking *Asc* I sites, while the pBR322 origin of replication can be replaced using the flanking *Swa* I sites. Any of the restriction sites shown in the MCS can be used for cloning your gene of interest; you must incorporate a stop codon in your PCR product if *the Xba* I site is used; since use of these sites may eliminate the existing stop codon (marked ***). Several MCS restriction sites are present in the same reading frame in both pEcoli-Nterm 6xHN and pEcoli-Cterm 6xHN vectors, namely *Stu* I, *Sal* I, *Bgl* II, *Hind* III, *Eco* RI and *Not* I. If these sites are used for cloning, the same insert can be used for both vectors. All restriction sites shown in the MCS, except for the *Pac* I sites, are unique. The pEcoli-Cterm 6xHN Linear (In-Fusion Ready) Vector (Figure 7) included in the TALON Express Bacterial Expression and Purification Kit is designed for convenient In-Fusion cloning using the *Sal* I and *Hind* III sites.

Appendix: Vector Information *continued*

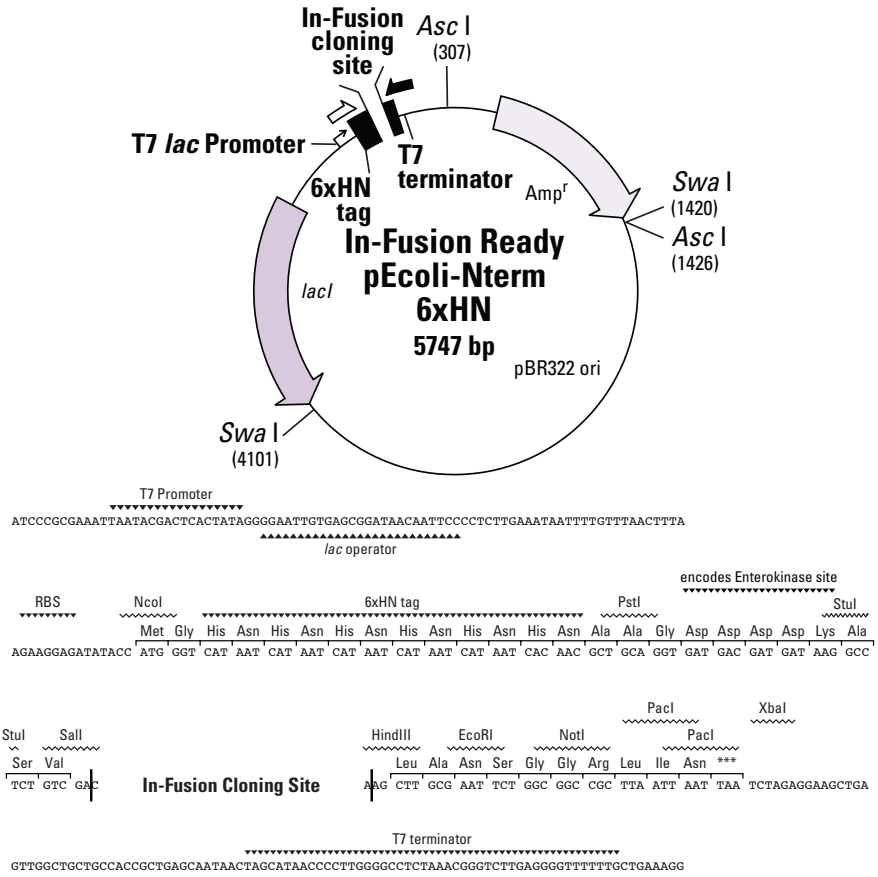

Figure 6. In-Fusion™ Ready pEcoli-Nterm 6xHN (Linear) Vector Map and MCS. In-Fusion Ready pEcoli-Nterm 6xHN (Linear) Vector is a prelinearized version of pEcoli-Nterm 6xHN designed for In-Fusion cloning. It has been digested using *Sal* I and *Hind* III, allowing easy cloning of PCR products in frame with the N-terminal 6xHN IMAC tag and adjacent enterokinase tag. The unshaded and black arrows on the left and right sides of the In-Fusion cloning site, respectively, represent the positions of the recommended forward (T7-Up1) and reverse (T7 terminator) sequencing primers (see Section VI.F for sequences). This highly inducible bacterial expression vector is based on the pET system (Dubendorff and Studier, 1991; Studier and Moffatt, 1986; Rosenberg et al., 1987, Studier et al., 1990). A derivative of pET11 (Dubendorff and Studier, 1991), it combines the strongly induced T7 promoter with an operator for the Lac repressor to produce tightly regulated expression. The vector expresses *lac* repressor from the *lacI* gene, which represses expression from the T7 promoter in the absence of the inducer IPTG. pEcoli-Nterm 6xHN also contains an ampicillin resistance marker and a pBR322 origin of replication, which maintains the plasmid at a low-copy-number to further reduce expression in the uninduced state. The ampicillin resistance marker (Amp^r) can be replaced using the flanking *Asc* I sites, while the pBR322 origin of replication can be replaced using the flanking *Swa* I sites. The vector has been linearized using *Sal* I and *Hind* III at the indicated "In-Fusion Cloning Site" and thus no longer contains the cytosine and adenosine residues shown inside the vertical lines. In order to maintain the correct reading frame and restore the *Sal* I and *Hind* III sites, these nucleotides should be incorporated into the In-Fusion primers. If you prefer not to recreate these sites, you may replace these in the primers with other nucleotides. If you wish to use other restriction sites in the MCS, we recommend using the circular version of pEcoli-Nterm 6xHN (Figure 4) included in the TALON Express Bacterial Expression and Purification Kit.

Appendix: Vector Information *continued*

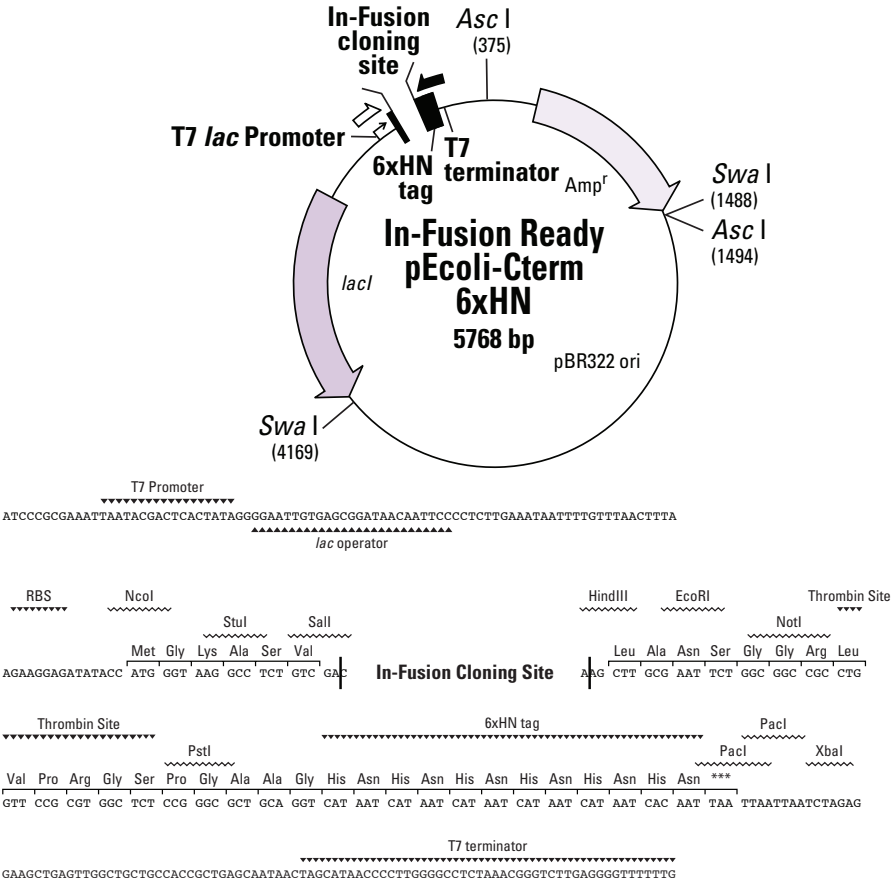

Figure 7. In-Fusion™ Ready pEcoli-Cterm 6xHN (Linear) Vector Map and MCS. In-Fusion Ready pEcoli-Cterm 6xHN (Linear) Vector is a prelinearized version of pEcoli-Cterm 6xHN designed for In-Fusion cloning. It has been digested using *Sal* I and *Hind* III, allowing easy cloning of PCR products in frame with the C-terminal 6xHN IMAC tag and adjacent thrombin tag. The unshaded and black arrows on the left and right sides of the In-Fusion cloning site, respectively, represent the positions of the recommended forward (T7-Up1) and reverse (T7 terminator) sequencing primers (see Section VI.F for sequences). This highly inducible bacterial expression vector is based on the pET system (Dubendorff and Studier, 1991; Studier and Moffatt, 1986; Rosenberg et al., 1987, Studier et al., 1990). A derivative of pET11 (Dubendorff and Studier, 1991), it combines the strongly induced T7 promoter with an operator for the Lac repressor to produce tightly regulated expression. The vector expresses *lac* repressor from the *lacI* gene, which represses expression from the T7 promoter in the absence of the inducer IPTG. pEcoli-Cterm 6xHN also contains an ampicillin resistance marker and a pBR322 origin of replication, which maintains the plasmid at a low-copy-number to further reduce expression in the uninduced state. The ampicillin resistance marker (Amp^r) can be replaced using the flanking *Asc* I sites, while the pBR322 origin of replication can be replaced using the flanking *Swa* I sites. The vector has been linearized using *Sal* I and *Hind* III at the indicated "In-Fusion Cloning Site" and thus no longer contains the cytosine and adenosine residues shown inside the vertical lines. In order to maintain the correct reading frame and restore the *Sal* I and *Hind* III sites, these nucleotides should be incorporated into the In-Fusion primers. If you prefer not to recreate these sites, you may replace these in the primers with other nucleotides. If you wish to use other restriction sites in the MCS, we recommend using the circular version of pEcoli-Cterm 6xHN (Figure 5) included in the TALON Express Bacterial Expression and Purification Kit.

Appendix: Vector Information *continued*

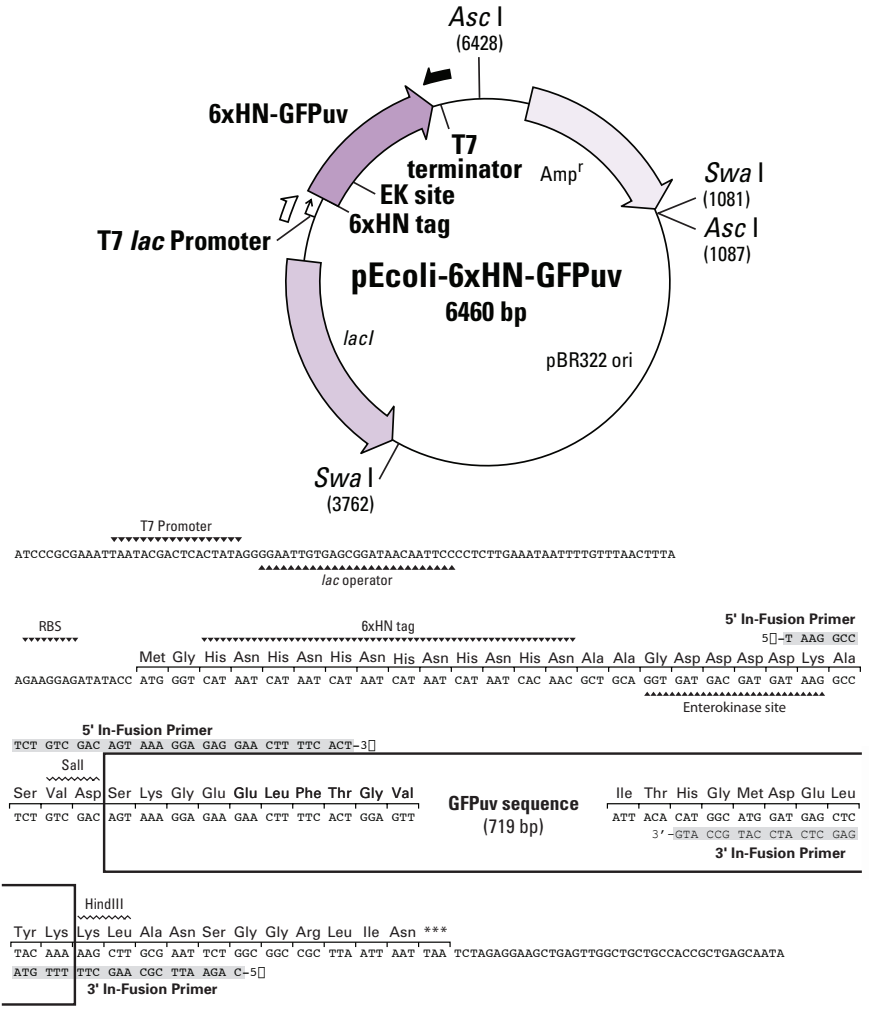

Figure 8. pEcoli-6xHN-GFPuv Vector Map and MCS. pEcoli-6xHN-GFPuv is a control vector expressing GFPuv with an N-terminal 6xHN IMAC tag and enterokinase site. This vector was created by cloning GFPuv into pEcoli-Nterm 6xHN at the *Sal* I and *Hind* III sites using In-Fusion cloning technology. The unshaded and black arrows on the left and right sides of the 6xHN-GFPuv gene, respectively, represent the positions of the recommended forward (T7-Up1) and reverse (T7 terminator) sequencing primers (see Section VI.F for sequences). This vector can be used to monitor protein expression and purification using the fluorescence of GFPuv, as well as by other methods suitable for protein analysis, including SDS-PAGE. The vector expresses Lac repressor from the *lacI* gene, which represses expression from the T7 promoter in the absence of the inducer IPTG. pEcoli-6xHN-GFPuv also contains an ampicillin resistance marker and a pBR322 origin of replication, which maintains the plasmid at a low-copy-number to further reduce expression in the uninduced state. The ampicillin resistance marker (Amp^r) can be replaced using the flanking *Asc* I sites, while the pBR322 origin of replication can be replaced using the flanking *Swa* I sites. The 5' end of the MCS and GFPuv coding sequences are shown, along with the encoded amino acids. The 6xHN tag (His-Asn)$_6$ and enterokinase sites are indicated, as is the sequence corresponding to GFPuv.

Notes

Notes

Notes

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The cycle 3 mutant GFP gene was produced by Maxygen, Inc. using DNA shuffling technology.

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