Preface

The aim of *E. coli Gene Expression Protocols* is to familiarize and instruct the reader with currently popular and newly emerging methodologies that exploit the advantages of using *E. coli* as a host organism for expressing recombinant proteins. The chapters generally fall within two categories: (1) the use of *E. coli* vectors and strains for production of pure, functional protein, and (2) the use of *E. coli* as host for the functional screening of large collections of proteins or peptides. These methods and protocols should be of use to researchers over a wide range of disciplines. Chapters that fall within the latter category describe protocols that will be particularly relevant for functional genomics studies.

The chapters of *E. coli Gene Expression Protocols* are written by experts who have hands-on experience with the particular method. Each article is written in sufficient detail so that researchers familiar with basic molecular techniques and experienced with handling *E. coli* and its bacteriophages should be able to carry out the procedures successfully. As in all volumes of the Methods in Molecular Biology series, each chapter includes an extensive Notes section, in which practical details peculiar to the particular method are described.

E. coli Gene Expression Protocols is not intended to be all inclusive, but is focused on new tools and techniques—or new twists on old techniques—that will likely be widely used in the coming decade. There are several well-established *E. coli* expression systems (e.g., the original T7 RNA polymerase expression strains and vectors developed by William F. Studier and colleagues; the use of GST and polyhistidine fusion tags for protein purification) that have been extensively described in other methods volumes and peer-reviewed journal articles and are thus not included in this volume, with the exception of a few contributions in which certain of these systems have been adapted for novel applications or otherwise improved upon.

It is my sincerest hope that both novice and seasoned molecular biologists will find *E. coli Gene Expression Protocols* a useful lab companion for years to come. I wish to thank all the authors for their excellent contributions and Prof. John M. Walker for sound advice and assistance throughout the editorial process.

Peter E. Vaillancourt

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Dual-Expression Vectors for Efficient Protein Expression in Both *E. coli* and Mammalian Cells

Rebecca L. Mullinax, David T. Wong, Heidi A. Davis, Kerstein A. Padgett, and Joseph A. Sorge

1. Introduction

In the near future, the nucleotide sequence of the genomes from many different organisms will be available. The next and more challenging step will be to characterize the biological role of each gene and the way in which the encoded protein functions in the cell. Dual-expression vectors for expression of proteins encoded by these genes in mammalian and bacterial cells can be used for this characterization. Typically, eukaryotic genes are expressed in mammalian cells to characterize biological functions and in bacterial cells to facilitate isolation of the protein. This generally requires the use of more than one vector. In contrast, use of a dual-expression vector eliminates the need to subclone from one vector system to another by combining the essential features of both eukaryotic and prokaryotic vectors in a single vector.

The pDual® GC expression system was designed for high-level protein expression in mammalian and bacterial cells (*see* **Fig. 1A**; ***[1,2]***). cDNA inserts encoding proteins are inserted into the vector using the unique seamless cloning method (*see* **Fig. 1B**; ***[5]***). This method is advantageous because it can result in the expression of the protein without extraneous amino acids encoded by restriction sites at the termini. As an alternative, the method allows for the optional expression of vector-encoded protein sequences that can be used to detect and purify the protein.

All pDual GC clones can express a fusion protein consisting of the cDNA, a thrombin cleavage site, three copies of the c-myc epitope tag, and a single copy of the 6xHis epitope and purification tag. The c-myc epitope is derived from the human c-*myc* gene and contains 10 amino acid residues (EQKLISEEDL;

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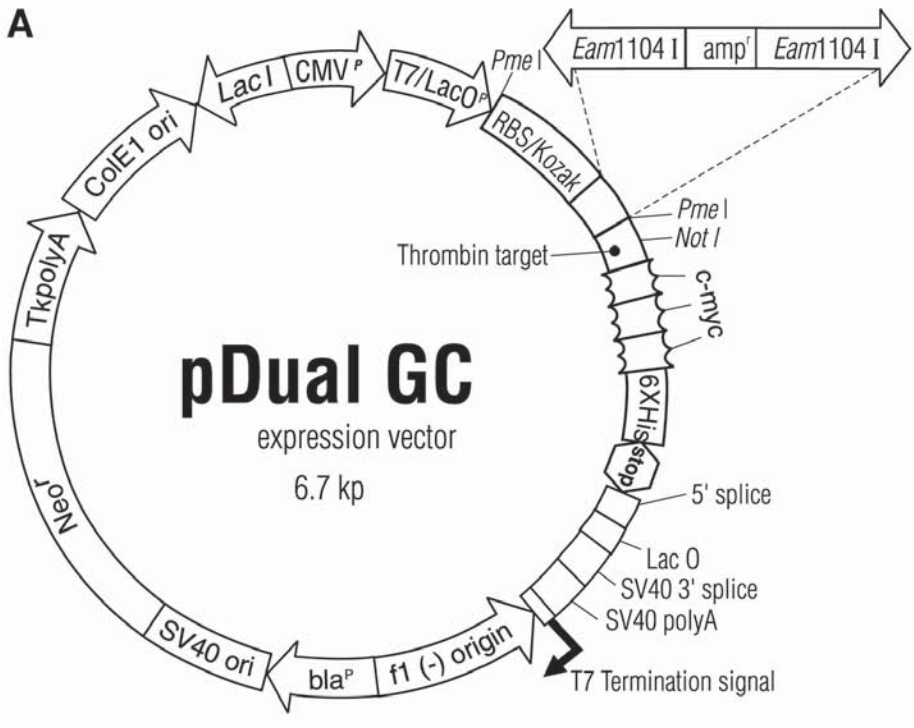

B

*Eam*1104 I

NN**CTCTTC**NATG
NN**GAGAAG**NTAC

cDNA insert

*Eam*1104 I

CTTN**GAAGAG**NN
GAAN**CTTCTC**NN

PCR product

*Eam*1104 I *Eam*1104 I

pDUAL GC --- ATGN**GAAGAG** ---------- **CTTCTC**NCTT --- pDUAL GC
pDUAL GC --- TACN**CTTCTC** ---------- **GAAGAG**NGAA --- pDUAL GC

pDUAL GC

digest with *Eam*1104 I

----- cDNA insert ------ CTT
TAC cDNA insert

PCR product

pDUAL GC --- ATG --- pDUAL GC
pDUAL GC --- GAA --- pDUAL GC

pDUAL GC

anneal and ligate

pDUAL GC --- ATG ----- cDNA insert -------- CTT --- pDUAL GC
pDUAL GC --- TAC ----- cDNA insert -------- GAA --- pDUAL GC

expression clone

[6]). This allows for sensitive detection and immunoprecipitation of expressed proteins with anti–c-myc antibody. The 6xHis epitope and purification tag consists of six histidine residues and allows for quick and easy detection of expressed proteins with anti-6xHis antibody and purification of the fusion protein from bacterial cells using a nickel-chelating resin ***(7)***. A thrombin cleavage site between the protein encoded by the cDNA and the c-myc and 6xHis tags allows for the removal of both tags when desired, for example, following protein purification.

A *Not*I recognition site is located between the cDNA insertion site and sequences encoding the thrombin cleavage site. This site allows for the insertion of nucleotides encoding protein domains that would be expressed as a C-terminal fusion to the expressed protein. An example would be to insert nucleotides encoding hrGFP ***(8)*** followed by a translational stop codon. The clone would then express

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Fig. 1. **(A)** The vector contains a mutagenized version of the promoter and enhancer region of the human cytomegalovirus (CMV) immediate early gene for constitutive expression of the clones in either transiently or stably transfected mammalian cells. Inducible gene expression in prokaryotes is directed from the hybrid T7/*lacO* promoter. The vector carries a copy of the *lac* repressor gene (*laqI*^{q}), which mediates tight repression of protein expression in the absence of the inducer, isopropyl-β-D-thiogalactopyranoside (IPTG). Expression is therefore regulated using IPTG in bacteria that express T7 polymerase under the regulation of the *lac* promoter. A tandem arrangement of the bacterial Shine-Dalgarno ***(3)*** and mammalian Kozak ***(4)*** ribosomal binding sites (RBS) allows for efficient expression of the open reading frame (ORF) in both bacterial and mammalian systems. In both bacterial and mammalian cells, the dominant selectable marker is the neomycin phosphotransferase gene, which is under the control of the β-lactamase promoter in bacterial cells and the SV40 promoter in mammalian cells. Expression of the neomycin phosphotransferase gene in mammalian cells allows stable clone selection with G418, whereas in bacteria the gene confers resistance to kanamycin. The *beta-lactamase* gene (amp^{r}), which confers resistance to ampicillin in bacteria, is removed during preparation of the expression clone. **(B)** The PCR product and pDUAL GC vector contain Eam*1104* I restriction sites (bold). Digestion of the PCR product and pDUAL GC vector with Eam*1104* I create complementary 3-base overhanging ends (underlined). Directional annealing of the complementary bases followed by ligation results in an expression clone capable of expressing the encoded cDNA in bacterial and mammalian cells. ATG, encoding methionine, is the first codon of the cDNA protein. CTT, encoding leucine, follows the last codon of the protein encoded by the cDNA insert and allows for expression of the downstream thrombin cleavage site, three copies of the c-myc epitope tag, and a single copy of the 6xHis epitope and purification tag. Alternatively, nucleotides encoding a stop codon follow the last codon of the protein encoded by the cDNA insert thereby terminating protein expression.

a fusion protein consisting of the cDNA and hrGFP. Expression of this fusion protein in mammalian cells allows for subcellular detection of the fusion protein.

A wide variety of proteins have been expressed using the pDUAL GC expression vector. To date, over 500 different eukaryotic proteins have been expressed in mammalian cells and detected using the c-myc epitope tag ***(9)***. In addition, over 100 different eukaryotic proteins have been expressed in bacterial cells and detected using the 6xHIS epitope tag (Ed Marsh, personal communication). These proteins are members of many different classes of proteins including kinases, DNA-binding proteins, transferases, transporters, oncogenes, cytochromes, proteases, inflammatory response proteins, cellular matrix proteins, metabolic proteins, synthases, esterases, zinc-finger proteins, and ribosomal proteins. Potential uses for these expressed proteins include analyzing protein function, defining both protein-protein and protein-DNA interactions, elucidating pathways, studying protein degradation, studying catalytic activity, determining the effects of over-expression, and preparing antigen.

2. Materials

2.1. Preparation of Plasmid Expressing Protein of Interest

2.1.1. Preparation of cDNA Insert

1. PCR primers containing *Eam*1104 I recognition sites.
2. DNA template encoding gene of interest.
3. Pfu DNA polymerase.
4. 10X Cloned Pfu polymerase buffer: 100 m*M* KCl, 100 m*M* $(NH_4)_2SO_4$, 200 m*M* Tris-HCl, pH 8.8, 20 m*M* $MgSO_4$, 1% Triton X-100, and 1 mg/mL of nuclease-free bovine serum albumin (BSA).
5. 5-Methyldeoxycytosine (^{m5}dCTP), optional.
6. *Eam*1104 I restriction enzyme.
7. 10X Universal buffer: 1 *M* potassium acetate (KOAc), 250 m*M* Tris-acetate, pH 7.6, 100 m*M* magnesium acetate ($Mg(OAc)_2$), 5 m*M* β-mercaptoethanol, and 100 µg/mL BSA. Autoclave.

2.1.2. Preparation of pDUAL GC Expression Vector

1. pDual® GC expression vector.
2. *Eam*1104 I restriction enzyme.
3. 10X Universal buffer: 1 *M* KOAc, 250 m*M* Tris-acetate, pH 7.6, 100 m*M* $Mg(OAc)_2$, 5 m*M* β-mercaptoethanol, and 100 µg/mL BSA. Autoclave.

2.1.3. Ligation of cDNA insert and pDUAL GC Expression Vector

1. T4 DNA ligase.
2. 10X Ligase Buffer: 500 m*M* Tris-HCl, pH 7.5, 70 m*M* $MgCl_2$, and 10 m*M* dithiothreitol (DTT).

3. T4 DNA ligase dilution buffer (1X ligase buffer).
4. 10 m*M* rATP.
5. Epicurian Coli® XL1-Blue supercompetent cells MRF' (Stratagene).
6. β-Mercaptoethanol.
7. SOB medium per liter: 20.0 g of tryptone, 5.0 g of yeast extract, and 0.5 g of NaCl. Autoclave. Add 10 mL of 1 *M* $MgCl_2$ and 10 mL of 1 *M* $MgSO_4$.
8. SOC medium per 100 mL: 1 mL of a 2 *M* filter-sterilized glucose solution or 2 mL of 20% (w/v) glucose. Adjust to a final volume of 100 mL with SOB medium. Filter sterilize.
9. Luria-Bertani (LB) agar per liter: 10 g of NaCl, 10 g of tryptone, 5 g of yeast extract, and 20 g of agar. Add dH_2O to a final volume of 1 L. Adjust pH to 7.0 with 5 N NaOH. Autoclave. Pour into Petri dishes (~25 mL/100-mm Petri dish).
10. LB-kanamycin agar per liter: Prepare 1 L LB agar. Autoclave. Cool to 55°C and add 5 mL of 10 mg/mL-filter-sterilized kanamycin. Pour into Petri dishes (~25 mL/100-mm plate).
11. LB broth per liter: 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract. Add deionized H_2O to a final volume of 1 L. Adjust pH to 7.0 with 5 *N* NaOH. Autoclave.
12. LB-kanamycin broth per liter: Prepare 1 L of LB broth. Autoclave. Cool to 55°C. Add 5 mL of 10 mg/mL-filter-sterilized kanamycin.
13. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, pH 7.5, and 1 m*M* ethylenediaminetetraacetic acid (EDTA). Autoclave.

2.2. Protein Expression in Bacterial Cells

1. *Escherichia coli* competent cells that express T7 polymerase in the presence of IPTG.
2. IPTG (1 *M*): 238.3 mg/mL in distilled water.
3. 2X Sodium dodecyl sulfate (SDS) gel sample buffer: 100 m*M* tris-HCl, pH 6.5, 4% (w/v) SDS (electrophoresis grade), 0.2% (w/v) bromophenol blue, and 20% (v/v) glycerol. Add dithiothreitol (DTT) to a final concentration of 200 m*M* before use.

3. Methods

Additional information regarding these techniques that is beyond the scope of this chapter can be found in **ref. *10***.

3.1. Design of Primers Used to Amplify cDNA Insert

The cDNA inserts are generated by PCR amplification with primers that contain *Eam*1104 I recognition sites and a minimal flanking sequence at their 5' termini. The ability of *Eam*1104 I to cleave several bases downstream of its recognition site allows the removal of superfluous, terminal sequences from the amplified DNA insert. The elimination of extraneous nucleotides and the generation of unique, nonpalindromic sticky ends permit the formation of directional seamless junctions during the subsequent ligation to the pDual GC expression vector.

The cDNA insert is amplified using PCR primers to introduce *Eam*1104 I recognition sites in each end of the cDNA insert to position the cDNA in the pDUAL GC expression vector for optimal protein expression. *Eam*1104 I is a type IIS restriction enzyme that has the capacity to cut outside its recognition sequence (5'-CTCTTC-3'). The cleavage site extends one nucleotide on the upper strand in the 3' direction and four nucleotides on the lower strand in the 5' direction. Digestion with *Eam*1104 I generates termini that feature three nucleotides in their 5' overhangs. A minimum of two extra nucleotides must precede the 5'-CTCTTC-3' recognition sequence in order to ensure efficient cleavage of the termini. The bases preceding the recognition site can be any of the four nucleotides.

The forward primer must be designed with one extra nucleotide (N) located between the *Eam*1104 I recognition sequence and the gene's translation initiation codon, in order to generate the necessary 5'-ATG overhang that is complementary to the pDUAL GC expression vector sequence. The forward primer should be designed to look as follows: 5'-NNCTCTTCNATG$(X)_{15}$-3'; where N denotes any of the four nucleotides, X represents gene-specific nucleotides, and the underlined nucleotides represent the *Eam*1104 I recognition site.

The reverse primer must be designed with one nucleotide (N) located between the *Eam*1104 I recognition sequence and the AAG triplet that comprises the 5' overhang complementary to the vector sequence. Depending on whether or not the c-myc and 6xHIS tags are desired as fusion partners, the reverse primer should be designed to look as follows: (1) Reverse primer design to allow the expression of the c-myc and 6xHIS fusion tags: 5'-NNCTCTTCNAAG$(X)_{15}$-3'; where N denotes any of the four nucleotides and X represents the gene-specific nucleotides. (2) The reverse primer design that does not allow expression of the c-myc and 6xHIS fusion tags: 5'-NNCTCTTCNAAG*TTA*$(X)_{15}$-3'; where N denotes any of the four nucleotides and X represents the gene-specific nucleotides. The necessary stop codon is shown in italics.

The primer should be complementary to a minimum of 15 nucleotides of the template on the 3' end of the PCR primer in addition to the *Eam*1104 I recognition sequence. The estimated T_m [$T_m \approx 2°C$ (A + T) + 4°C (G + C)] of the homologous portion of the primer should be 55°C or higher, with a G-C ratio of 60% or more.

3.2. PCR Amplification of cDNA Insert0

If the insert contains an internal *Eam*1104 I recognition site, the amplification reaction should be performed in the presence of 5-methyldeoxycytosine triphosphate (^{m5}dCTP) for the last five cycles of the PCR (*see* **Note 1**). Incorporation of ^{m5}dCTP during the PCR amplification protects already-existing internal *Eam*1104 I sites from subsequent cleavage by the endonuclease ***(1,2,5)***.

The primer-encoded *Eam*1104 I sites are not affected by the modified nucleotide because the newly synthesized strand does not contain cytosine residues in the recognition sequence.

1. Combine the following components in a 500–µL thin-walled tube (*see* **Note 2**). Add the components in the order given. Mix the components well before adding the Pfu DNA polymerase (*see* **Note 3**): 81.2 µL distilled water, 10.0 µL 10X Pfu DNA polymerase buffer, 0.8 µL 25 m*M* each dNTP, 1.0 µL 1–100 ng/µL plasmid DNA template, 2.5 µL 10 µM primer #1, 2.5 µL 10 µ*M* primer #2, and 2.0 µL 2.5 U/µL cloned Pfu DNA polymerase.
2. Recommended cycling parameters
   a. For inserts that do not contain internal *Eam*1104 I restriction sites (*see* **Note 4**): 1 cycle at 94–98°C, 45 s; 25–30 cycles at 94–98°C, 45 s; primer T_m –5°C, 45 s; and 72°C for 1–2 min/kb of PCR target; and 1 cycle at 72°C, 10 min.
   b. For inserts that contain internal *Eam*1104 I restriction sites: (*see* **Notes 4** and **5**) 1 cycle at 94–98°C, 45 s; 20–25 cycles at 94–98°C, 45 s; primer T_m –5°C, 45 s; and 72°C for 1–2 min/kb of PCR target and 1 cycle at 72°C, 10 min. After the first PCR, add 1 µL 25 m*M* ^{m5}dCTP. Perform a second PCR of 5 cycles at 98°C, 45 s; primer T_m –5°C, 45 s; and 72°C for 1–2 min/kb of PCR target and 1 cycle at 72°C, 10 min.
3. Analyze the PCR amplification products on a 0.7–1.0% (w/v) agarose gel.

3.3. PCR Product Purification

Before digestion, the PCR product must be removed from unincorporated PCR primers, unincorporated nucleotides, and the thermostable polymerase. Suitable purification methods include phenol:chloroform extraction, selective precipitation gel purification, or spin-cup purification. To prepare the insert for ligation, treat the PCR product with *Eam*1104 I (≥24 units/µg PCR product).

1. Mix the following components in a 1.5-mL microcentrifuge tube: dH_2O for a final volume of 30 µL, 1–5 µL of PCR product, 3 µL of 10X universal buffer, and 3 µL of 8 U/µL *Eam*1104 I restriction enzyme.
2. Mix the digestion reaction gently and incubate at 37°C for 1 h.
3. Purify the digested PCR product by gel purification (*see* **Note 6**) and resuspend in TE buffer.

3.4. Eam*1104 I Digestion of pDual GC Expression Vector*

The cloning region of the pDual GC expression vector is characterized by the presence of two *Eam*1104 I recognition sequences (5'-CTCTTC-3') directed in opposite orientations and separated by a spacer region. The sites are positioned for maximal protein expression and optional expression of the downstream epitope and purification tags. Digestion with *Eam*1104 I restriction enzyme creates 3-nucleotide 5' overhangs that are directionally ligated to the 5' overhangs of the cDNA insert. Because one of the sticky ends in the pDUAL

GC expression vector is complementary to the ATG of the cDNA insert, protein expression begins with the gene's own translation initiation codon. Digestion of the pDUAL GC expression vector creates two nonpalindromic, nonidentical overhanging ends and results in directional ligation of the cDNA insert.

To generate a ligation-ready vector for PCR cloning, the pDual GC expression vector is digested with *Eam*1104 I.

1. Mix the following components in a 1.5-mL microcentrifuge tube: dH_2O for a final volume of 30 µL, ≤1 µg pDUAL GC expression vector (*see* **Note 7**), 3 µL of 10X universal buffer and 3 µL of 8 U/µL *Eam*1104 I restriction enzyme.
2. Mix the digestion reaction gently and incubate at 37°C for 2 h.
3. Purify the digested vector by gel purification and resuspend in TE buffer to a final concentration of 100 ng/µL.

3.5. Ligation of Digested Vector and Insert

The vector and insert are directionally ligated at the compatible overhanging ends.

1. Combine the following in a 1.5-mL microcentrifuge tube: 1 µL 100 ng/µL digested pDUAL GC expression vector, x µL digested insert (3:1 molar ratio of insert to vector, *see* **Note 8**), 2 µL 10X ligase buffer, 2 µL of 10 m*M* rATP, 1 µL of (4 U/µL) T4 DNA ligase, and dH_2O to a final volume of 20 µL.
2. Mix the ligation reactions gently and then incubate for 1 h at room temperature or overnight at 16°C.
3. Store the ligation reactions on ice until ready to use for transformation into *E. coli* competent cells.

3.6. Transformation of Ligated DNA

Methylation of nucleic acids has been found to affect transformation efficiency. If the cDNA insert was amplified in the presence of methylated dCTP (^{m5}dCTP), use an *E. coli* strain that does not have an active restriction system that restricts methylated cytosine sequences, such as Epicurian Coli® XL1-Blue MRF' supercompetent cells (Stratagene).

1. Prepare competent cells and keep on ice.
2. Gently mix the cells by hand. Aliquot 100 µL of the cells into a prechilled 15-mL Falcon 2059 polypropylene tube.
3. Add 1.7 µL of the 14.2 *M* β-mercaptoethanol to 100 µL of bacteria.
4. Swirl the contents of the tube gently. Incubate the cells on ice for 10 min, swirling gently every 2 min.
5. Add 5 µL of the ligation reaction to the cells and swirl gently.
6. Incubate the tubes on ice for 30 min.
7. Prepare and equilibrate SOC medium to 42°C.

8. Heat-pulse the tubes in a 42°C water bath for 45 s. The length of time of the heat pulse is critical for obtaining the highest efficiencies.
9. Incubate the tubes on ice for 2 min.
10. Add 0.9 mL of equilibrated SOC medium and incubate the tubes at 37°C for 1 h with shaking at 225–250 rpm (*see* **Note 9**).
11. Using a sterile spreader, plate 5–10% of the transformation reactions onto separate LB-kanamycin agar plates.
12. Incubate the plates overnight at 37°C.
13. Identify colonies containing the desired clone by isolation of miniprep DNA from individual colonies followed by restriction enzyme analysis. Determining the nucleotide sequence of the cDNA insert is highly recommended.

3.7. Protein Expression and Detection in Bacterial Cells

For expression of the fusion protein in bacteria, transform mini-prep DNA into *E. coli* cells which express T7 polymerase when induced with IPTG (*see* **Note 10**). The following is a small-scale protocol intended for the analysis of individual transformants.

1. Prepare competent cells.
2. Transform competent cells with pDUAL GC clone.
3. Identify colonies containing pDUAL GC clone.
4. Inoculate 1-mL aliquots of LB broth (containing 100 µg/mL ampicillin) with single colonies (*see* **Note 11**). Incubate at 37°C overnight with shaking at 220–250 rpm.
5. Transfer 100 µL of each overnight culture into fresh 1-mL aliquots of LB broth without antibiotics. Incubate at 37°C for 2 h with shaking at 220–250 rpm.
6. Transfer 100 µL of each 2-h culture into a clean microfuge tube and place the tube on ice until needed for gel analysis. These samples will be the noninduced control samples.
7. Add IPTG to a final concentration of 1 m*M* to the remaining 2-h cultures. Incubate at 37°C for 4 h with shaking at 220–250 rpm (*see* **Notes 12–13**).
8. At the end of the incubation period, place the induced cultures on ice.
9. Pipet 20 µL of each induced culture into a clean microcentrifuge tube. Add 20 µL of 2X SDS gel sample buffer to each tube.
10. Harvest the cells by centrifugation at 4000*g* for 15 min.
11. Decant the supernatant and store the cell pellet at –70°C if desired or process immediately to purify the induced protein.
12. Mix the tubes containing the non-induced cultures to resuspend the cells. Pipet 20 µL from each tube into a fresh microcentrifuge tube. Add 20 µL of 2X SDS gel sample buffer to each tube.
13. Heat all tubes to 95°C for 5 min and place on ice. Load samples on 6% SDS-PAGE gel with the noninduced samples and induced samples in adjacent lanes. Separate the proteins by electrophoresis at 125 V until the bromophenol blue reaches the bottom of the gel.

14. Stain the separated proteins in the gel using Coomassie® Brilliant Blue.
15. The amount of induced protein should be greater in the induced cultures than in the noninduced cultures.

3.8. Detection and Isolation of Protein from Bacterial Cells

Expression of the fusion protein containing the 6xHIS tag can be detected by Western blot analysis ***(10)*** using an anti-6xHIS antibody and isolated from the induced cultures by nickel metal affinity chromatography ***(7)***. The most commonly used reagent for isolating 6xHIS-tagged proteins is Ni-NTA (nickel nitrilotriacetic acid, QIAGEN).

3.9. Protein Expression and Detection in Mammalian Cells

Transfection of genes into mammalian cells for protein expression is a fundamental tool for the analysis of gene function. There are many well-established protocols that result in a high number of viable cells expressing the protein. These protocols include diethyl amino ethyl (DEAE)-dextran- phosphate ***(11)*** and calcium-mediated transfection ***(12)***. Many factors contribute to transient and stable transfection efficiency; however, the primary factor is the cell type. Different cell lines vary by several orders of magnitude in their ability to take up and express protein from plasmids. Other factors that effect efficiency include the use of highly purified plasmid DNA, optimal cell density, optimal transfection reagent to DNA ratio, and the optimal time the transfection reagent is in contact with the cells prior to dilution with growth medium. These conditions vary with each cell type.

Expression of the fusion protein containing the c-myc epitope can be detected in mammalian cell lysates by Western blot analysis ***(10)*** using an anti-c-myc antibody.

4. Notes

1. The addition of the ^{m5}dCTP is delayed until the final five cycles of amplification to avoid the possible deamination of the ^{m5}dCTP by extended exposure to cycles of heating and cooling.
2. The use of thin-wall tubes is highly recommended for optimal thermal transfer during PCR.
3. The use of a high fidelity polymerase, such as Pfu DNA polymerase, in the amplification reaction is highly recommended to eliminate mutations that could be introduced during the PCR. In addition, Pfu DNA polymerase is very thermostable and is not inactivated by the high temperatures used in this protocol.
4. Critical optimization parameters for successful amplification of the template DNA include the use of an extension time that is adequate for full-length DNA synthesis, sufficient enzyme concentration, optimization of the reaction buffer, adequate primer-template purity, and concentration and optimal primer design. Extension time is the most critical parameter affecting the yield of PCR product

obtained using Pfu DNA polymerase. The minimum extension time should be 1–2 min/kilobase pair of amplified template.

5. Thermal cycling parameters should be chosen carefully to ensure the shortest denaturation times to avoid enzyme inactivation, template damage and deamination of the ^{m5}dCTP, adequate extension times to achieve full-length target synthesis, and the use of annealing temperatures near the primer melting temperature to improve specificity of the PCR product.
6. Gel purification of the digested insert is optional but will reduce the number of colonies containing vector without insert following transformation.
7. Dephosphorylation of the vector is not required because nonidentical, nonpalindromic sticky ends are generated by the type IIS *Eam*1104 I restriction endonuclease.
8. The ideal insert-to-vector DNA ratio is variable; however, a reasonable starting point is 3:1 (insert-to-vector molar ratio), measured in available picomole ends. This is calculated as follows:

$$\text{picomole ends/microgram of DNA} = \frac{2 \times 106}{\text{number of base pair} \times 660}$$

9. Expression of the kanamycin gene by incubation of transformed cells in LB broth for at least 1 h prior to selection on LB-kanamycin agar plates is essential for efficient transformation.
10. The use of bacterial cells that express tRNA that are rare in *E. coli* but frequent in mammalian proteins is also highly recommended. Use of bacterial cells that are deficient in proteases, such as Lon and OmpT proteases, is highly recommended. These proteases can cause degradation of the over-expressed protein. The BL21-CodonPlus® competent cells contain extra copies of the argU, ileY, leuW, and/or proL tRNA, and are Lon and OmpT protease deficient. These tRNA are frequently of low abundance in *E. coli* cells but may be required for efficient translation of mammalian proteins. BL21-CodonPlus cells express T7 polymerase whose expression is induced in the presence of IPTG.
11. If BL21-CodonPlus cells are used, add 50 µg/mL chloramphenicol to the LB with ampicillin. Chloramphenicol is required to maintain the pACYC plasmid, which expresses the tRNA, in the BL21-Codon Plus strain.
12. The IPTG concentration and the induction time are starting values and may require optimization for each gene expressed.
13. The volume of induced culture required is determined by the protein expression level, protein solubility, and purification conditions. For proteins that are expressed at low levels, the minimum cell culture volume should be 50 mL.

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