Affinity® Protein Expression and Purification System and Affinity® Protein Expression Vectors

INSTRUCTION MANUAL

Affinity® Protein Expression and Purification Systems

#204301 (with pCAL-c Vector)

#204302 (with pCAL-n Vector)

Affinity® Protein Expression Vectors

#214301 (pCAL-c)

#214302 (pCAL-n)

#214310 (pCAL-n-EK)

#214311 (pCAL-n-FLAG)

Revision #045007h

For In Vitro Use Only

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Affinity® Protein Expression and Purification System and Affinity® Protein Expression Vectors

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Affinity® Protein Expression and Purification System and Affinity® Protein Expression Vectors

MATERIALS PROVIDED

Affinity® Protein Expression and Purification Systems

Materials provided	Catalog #204301	Catalog #204302
pCAL-c vector (1.0 µg/µl)	20 µg of DNA[a]	—
pCAL-n vector (1.0 µg/µl)	—	20 µg of DNA[a]
pTC12 control plasmid	10 µg	10 µg
BL21-Gold (DE3) competent cells[b]	10 × 0.1-ml aliquots	10 × 0.1-ml aliquots
pUC18 control plasmid (0.1 ng/µl in TE buffer)	10 µl	10 µl
Thrombin[c]	10 µg	10 µg
Calmodulin (CaM) affinity resin[d]	5 ml	5 ml
EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (100 mM)]	1 ml	1 ml

[a] Cesium chloride-banded, supercoiled plasmid DNA.

[b] Efficiencies are ≥1 × 10^8 transformants/µg.

[c] To avoid repeated freeze–thaw cycles, aliquot the stock solution into separate tubes and store at –80°C.

[d] Shipped and stored in 20% (v/v) ethanol.

Affinity® Protein Expression Vectors

Vector	Catalog #214300	Catalog #214301	Catalog #214302	Catalog #214310	Catalog #214311
pCAL-c	—	20 µg (1.0 µg/µl)	—	—	—
pCAL-n	—	—	20 µg (1.0 µg/µl)	—	—
pCAL-n-EK	—	—	—	20 µg (1.0 µg/µl)	—
pCAL-n-FLAG	—	—	—	—	20 µg (1.0 µg/µl)
pTC12 control plasmid	10 µg	10 µg	10 µg	10 µg	10 µg
XL1-Blue strain	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Note *The pCAL-c, pCAL-n, pCAL-n-EK, and pCAL-n-FLAG vector sequences are available from the GenBank® database (Accession #U36452, #U36453, #U36454, #U86347, and #AF087042 respectively).*

Revision #045007h

STORAGE CONDITIONS

BL21-Gold (DE3) Competent Cells: –80°C
pUC 18 (Control Plasmid): –80°C
XL1-Blue Strain: –80°C
Thrombin: –80°C
Affinity® Protein Expression Vectors: –20°C
pTC12 Control Plasmid: –20°C
EGTA: –20°C
Calmodulin Affinity Resin: 4°C

ADDITIONAL MATERIALS REQUIRED

Isopropyl-1-thio-β-D-galactopyranoside (IPTG)
Falcon® 2059 polypropylene tubes (15-ml)
Calf intestinal alkaline phosphatase (CIAP)
CIAP buffer (10×) (see *Preparation of Media and Reagents*)
Disposable columns and glass rods for pouring the degassed resin
Deoxynucleoside triphosphate
Primers
Gel purification system

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2. No materials that contain the cloned copy of T7 gene 1, the gene for T7 RNA polymerase, may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this license and agrees to be bound by its terms. This limitation applies to strain BL21-Gold(DE3) included in this kit and any derivatives you may make of it.

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INTRODUCTION

The Affinity® protein expression and purification system[1,2] allows simple, rapid, and efficient purification of calmodulin-binding-peptide (CBP)-tagged fusion proteins from *E. coli* extracts. The Affinity protein expression vectors pCAL-n, pCAL-n-EK, pCAL-n-FLAG, and pCAL-c, allow fusion of the CBP affinity tag[3,4] to the N or C terminus of the protein-coding sequence of interest. Protein expression is tightly repressed under conditions in which expression is undesirable, and high-level induced expression can be achieved. Calmodulin-binding-peptide fusion proteins can be purified from crude cell extracts to near homogeneity with one pass through calmodulin (CaM) affinity resin using moderate buffer conditions at neutral pH.[5]

The CBP affinity tag is based on the relatively high affinity ($K_d = 10^{-9}$) for CaM exhibited by a 26-amino-acid C-terminal fragment from muscle myosin light-chain kinase at physiological pH in the presence of calcium. When calcium is removed from the environment, CaM undergoes a conformational change that results in the release of its ligand (see Figure 1). The CBP affinity tag in the Affinity protein expression and purification system binds CaM with high affinity while maintaining gentle binding and elution conditions. The relatively small size of the 4-kDa CBP affinity tag is less likely to affect the function of the protein of interest than many affinity-tag systems currently in use. The Affinity vectors contain recognition sites for the site-specific proteases thrombin or enterokinase (EK) for proteolytic removal of affinity tags from purified proteins.

The Affinity protein expression and purification system includes one of three Affinity protein expression vectors, high-efficiency BL21-Gold (DE3) competent cells for protein expression, CaM affinity resin, and thrombin. The five Affinity vectors are available separately. Stratagene also offers the Affinity CBP fusion protein detection kit for detection of ≥10 ng of CBP fusion protein.

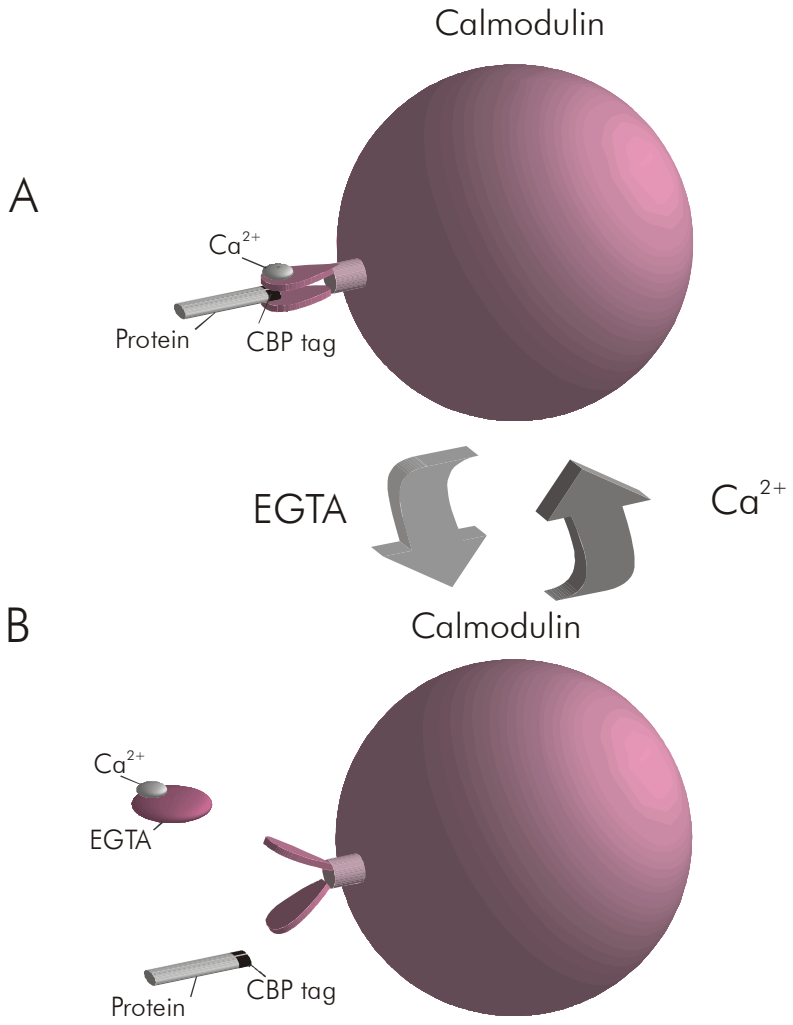


FIGURE 1 The Affinity protein expression and purification system. The highly conserved protein calmodulin binds to the CBP-tagged fusion protein in the presence of low concentrations of calcium at neutral pH (A). The fusion protein elutes from its ligand at neutral pH with 2 mM EGTA (B). The purified protein is now ready for storage, or if desired, proteolytic cleavage by thrombin or EK.

THE AFFINITY® PROTEIN EXPRESSION VECTORS

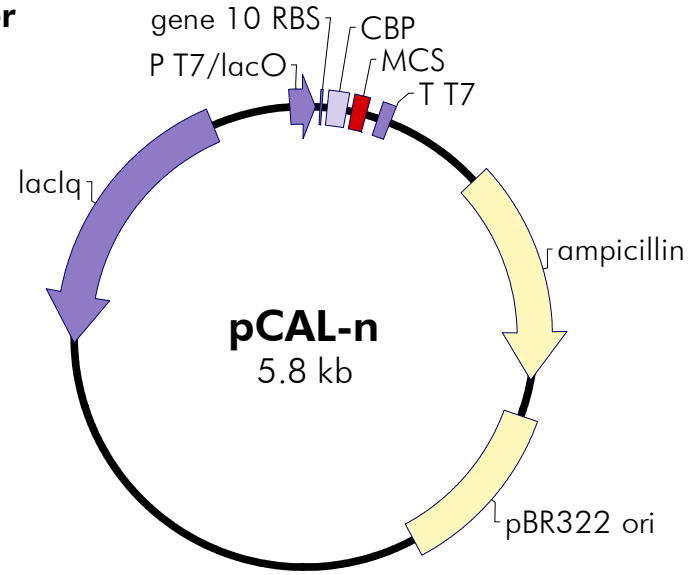
The Affinity protein expression vectors, pCAL-n, pCAL-n-EK, pCAL-n-FLAG, and pCAL-c (see Figures 2–5), are derived from the pET-11 vector series. The vectors are engineered to take advantage of the features of the bacteriophage T7 gene *10* promoter and leader sequence that allow high selectivity of the promoter by T7 RNA polymerase, tight repression in the uninduced state, and high-level expression upon induction.[7,8] The Affinity vectors use the T7 *lac* promoter configuration and carry a copy of the *lacI*^{q} gene to mediate this tight repression. The pTC12 vector is included as a positive control for induction and purification of CBP fusion proteins.

The pCAL-n vector, based on the pET-11a vector, carries the CBP-coding sequence inserted upstream of a multiple cloning site (MCS) to allow for the fusion of the CBP affinity tag at the N-terminus of the cloned protein-coding sequence The efficient translation of the CBP tag in *E. coli* ensures that fusion proteins containing the CBP at the N terminus will be consistently expressed at high levels. The recognition sequence for thrombin is inserted between the CBP-coding sequence and the MCS. Digestion of purified fusion protein with thrombin occurs between the arginine and glycine residues within the thrombin recognition sequence. (Note that the ***Xba* I** site in the MCS of the pCAL-n vector is not unique.) The pCAL-n-EK vector is a modified version of the pCAL-n vector that allows removal of the N-terminal fusion by cleavage with enterokinase. The pCAL-n-FLAG vector is identical to the pCAL-n-EK vector with the addition of the FLAG epitope between the CBP purification tag and the MCS.

The pCAL-c vector, based on the pET-11d vector, contains the thrombin target–CBP affinity tag located 3´ to the cloning sites for fusion of the affinity tag to the C terminus of the protein-coding sequence of interest. Inserts are cloned between the *Nco* I site, which contains an ATG positioned for optimal translation from the T7 gene *10* ribosome-binding site (RBS), and the *Bam*H I site. Thrombin digestion of proteins expressed from the Affinity vector results in the retention of the four N-terminal amino acids (MYPR) from the thrombin recognition sequence. Bi-directional cloning of inserts into the *Bam*H I site of pCAL-c allows fusion of the efficiently translated T7 gene *10* leader peptide to the N-terminus of the protein of interest.

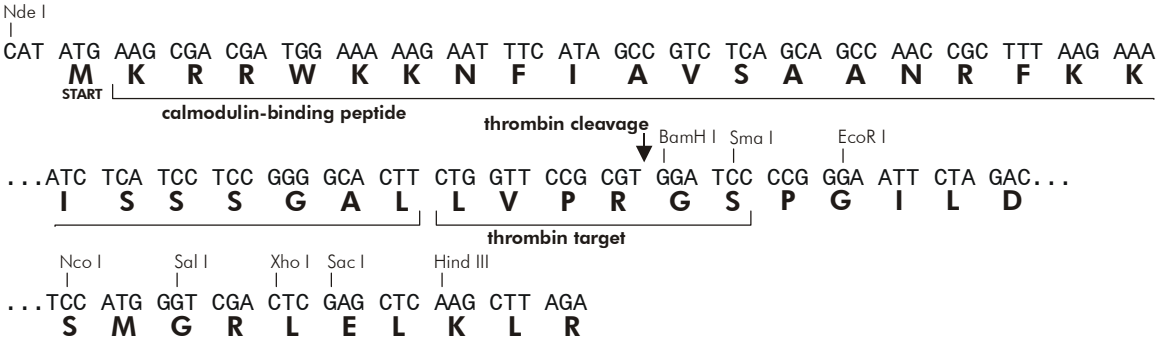
Caution *The T7 gene* 10 *leader and the C-terminal fusion tags, beginning with the Gly-Ser residues encoded by the* BamH *I restriction site, are in* ***separate frames****. When cloning bi-directionally into the* BamH *I restriction site, care should be taken that the protein coding sequence of interest is fused in frame with both the T7 gene* 10 *leader and the C-terminal fusion tag. When cloning bi-directionally into* Nco *I or* Nhe *I, the inserted amino acid sequence should be in frame with the C-terminal fusion tag beginning with the Gly-Ser residues encoded by the* BamH *I site.*

The pCAL-n Vector



pCAL-n Multiple Cloning Site Region

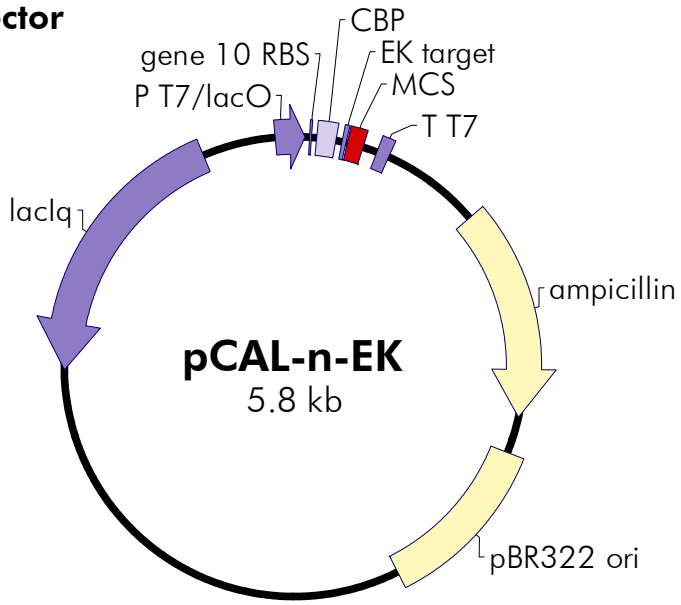
sequence shown (86–232)



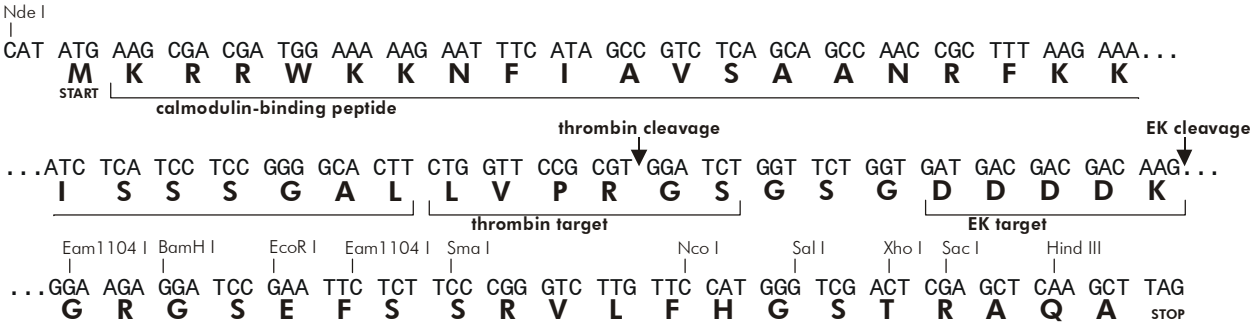
Feature	Nucleotide Position
T7 promoter with *lac* operator	1–44
T7 gene 10 ribosome binding site	74–80
calmodulin binding peptide (CBP)	92–169
thrombin target	170–187
multiple cloning site	182–229
T7 terminator	299–350
ampicillin resistance (*bla*) ORF	762–1619
pBR322 origin of replication	1770–2437
lacI^{q} repressor ORF	4317–5396

FIGURE 2 The pCAL-n vector

The pCAL-n-EK Vector



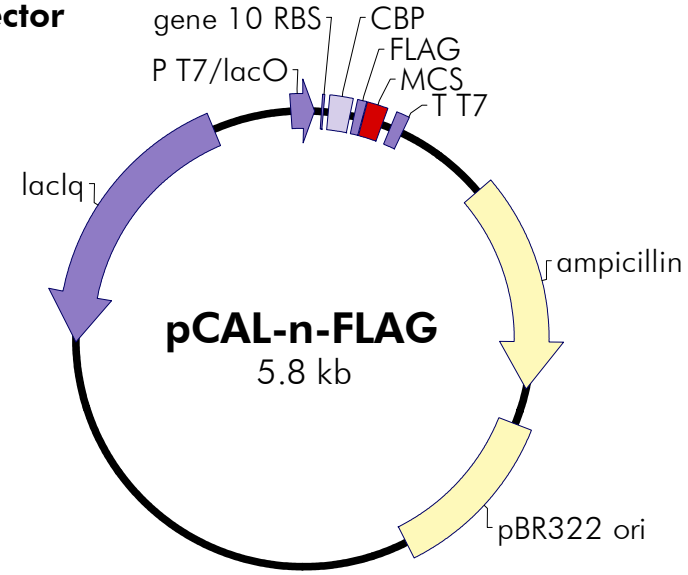
pCAL-n-EK Multiple Cloning Site Region
sequence shown (86–274)



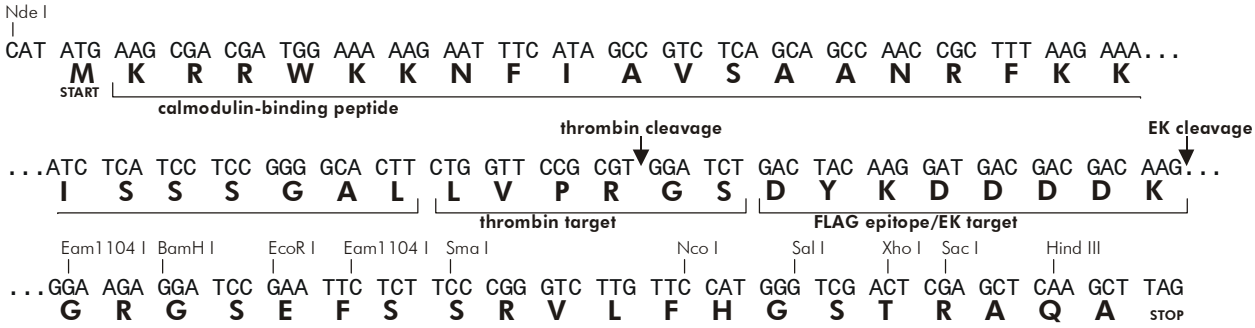
Feature	Nucleotide Position
T7 promoter with *lac* operator	1–44
T7 gene 10 ribosome binding site	74–80
calmodulin binding peptide (CBP)	92–169
thrombin target	170–187
enterokinase (EK) target	197–211
multiple cloning site	218–272
T7 terminator	342–393
ampicillin resistance (*bla*) ORF	805–1662
pBR322 origin of replication	1813–2480
lacI^{q} repressor ORF	4360–5439

FIGURE 3 The pCAL-n-EK vector

The pCAL-n-FLAG Vector



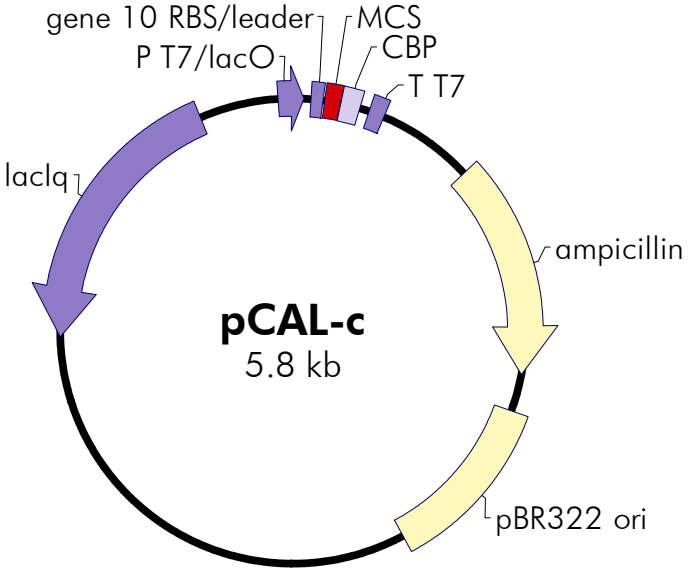
pCAL-n-FLAG Multiple Cloning Site Region
sequence shown (86–274)



Feature	Nucleotide Position
T7 promoter with *lac* operator	1–44
T7 gene 10 ribosome binding site	74–80
calmodulin binding peptide (CBP)	92–169
thrombin target	170–187
FLAG tag	188–211
enterokinase (EK) target	197–211
multiple cloning site	218–272
T7 terminator	342–393
ampicillin resistance (*bla*) ORF	805–1662
pBR322 origin of replication	1813–2480
lacI^{q} repressor ORF	4360–5439

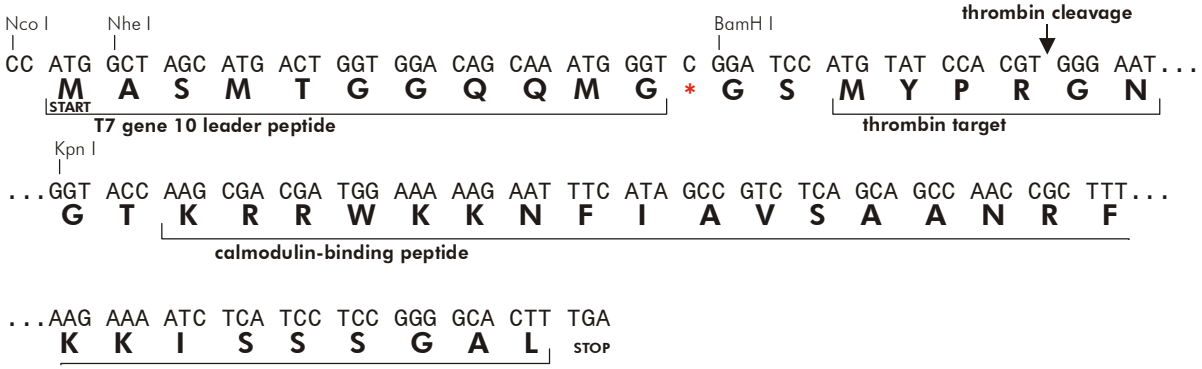
FIGURE 4 The pCAL-n-FLAG vector

The pCAL-c Vector



pCAL-c Multiple Cloning Site Region

sequence shown (86–232)



*ATG is not in frame with the C-terminal fusion tags.

Feature	Nucleotide Position
T7 promoter with *lac* operator	1–44
T7 gene 10 ribosome binding site/ translated leader	74–120
multiple cloning site	86—151
thrombin target	128–145
calmodulin binding peptide (CBP)	152–229
T7 terminator	299–350
ampicillin resistance (*bla*) ORF	762–1619
pBR322 origin of replication	1770–2437
lacI^{q} repressor ORF	4317–5396

FIGURE 5 The pCAL-c vector

TABLE 1

Vector Primers and Coordinates

Vector	Primer	Coordinates	Feature (encompassing site)
pCAL-c	pET 5' 19-mer	1–19	T7 promoter
	pCAL-c 3' 21-mer	169–189	Calmodulin Binding Peptide
pCAL-n	pCAL-n 5' 21-mer	109–129	Calmodulin Binding Peptide
	pET 3' 18-mer	280–297	T7 terminator
pCAL-n-EK	pCAL-n 5' 21-mer	109–129	Calmodulin Binding Peptide
	pET 3' 18-mer	323–340	T7 terminator
pCAL-n-FLAG	pCAL-n 5' 21-mer	109–129	Calmodulin Binding Peptide
	pET 3' 18-mer	323–340	T7 terminator

BL21(DE3) EXPRESSION STRAIN

The BL21(DE3) expression strain is derived from the *E. coli* B strain BL21, a strain that is generally good for protein expression due to its deficiency in *lon* protease as well as the *ompT* outer membrane protease that can degrade proteins during purification.[9–11] This strain is rifampicin sensitive (Rip^s), allowing use of the drug to inhibit transcription of host cell polymerase in instances where background synthesis is undesirable. The BL21(DE3) strain[7,9] carries a lambda DE3 lysogen that has the phage 21 immunity region, the *lacI* gene, and the *lacUV5*-driven T7 RNA polymerase expression cassette. On induction with IPTG, the *lacUV5* promoter is derepressed, allowing overexpression of T7 RNA polymerase and expression of the T7-promoted target gene from the pCAL-n-FLAG vector.

The BL21-Gold-derived expression strains incorporate major improvements over the original BL21 strain. The BL21-Gold strains feature the Hte phenotype present in Stratagene's highest efficiency strain, XL10-Gold® ultracompetent cells.[12] The presence of the Hte phenotype increases the transformation efficiency of the BL21-Gold cells to $\geq 1 \times 10^8$ cfu/µg of pUC18 DNA. In addition, the gene that encodes endonuclease I (*endA*), which rapidly degrades plasmid DNA isolated by most miniprep procedures, is inactivated. These two improvements allow direct cloning of many protein expression constructs.

Many genes that are expressed from the very strong T7 promoter can be toxic to the *E. coli* host cells. When using the BL21-Gold(DE3) strain as the primary host strain for cloning, some caution should be exercised because even low-level expression can result in accumulation of a toxic gene product. When the gene to be expressed is suspected of being host-lethal, Stratagene recommends either transforming BL21-Gold cells with the gene of interest (then inducing expression with CE6 bacteriophage) or using a general strain (e.g., XL1-Blue competent cells) for cloning and then transforming BL21-Gold(DE3)pLysS cells with miniprep DNA for expression.

Host Strain Genotypes

Host strain	Genotype
BL21 strain	*E. coli* B F$^-$ *dcm ompT hsdS*(r_B-m_B-) *gal*
BL21(DE3) strain	*E. coli* B F$^-$ *dcm ompT hsdS*(r_B-m_B-) *gal* λ (DE3)
BL21(DE3)pLysS strain	*E. coli* B F$^-$ *dcm ompT hsdS*(r_B-m_B-) *gal* λ (DE3) [pLysS Cam^r]
BL21-Gold	*E. coli* B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal endA* Hte
BL21-Gold (DE3)	*E. coli* B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte
BL21-Gold (DE3)pLysS	*E. coli* B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [pLysS Cam^r]

In order to further reduce basal activity of T7 RNA polymerase in the uninduced state, the BL21(DE3)pLysS strain carries a low-copy-number plasmid that carries an expression cassette from which the T7 lysozyme gene is expressed at low levels. T7 lysozyme binds to T7 RNA polymerase and inhibits transcription by this enzyme. On IPTG induction, overproduction of the T7 RNA polymerase renders low-level inhibition by T7 lysozyme virtually ineffective. In addition to inactivation of T7 RNA polymerase transcription, T7 lysozyme has a second function involving specific cleavage of the peptidoglycan layer of the *E. coli* outer wall. The inability of T7 lysozyme to pass through the bacterial inner membrane restricts the protein to the cytoplasm, allowing *E. coli* to tolerate expression of the protein. This second function of lysozyme confers the further advantage of allowing cell lysis under mild conditions. Cells expressing T7 lysozyme are subject to lysis under conditions that would normally only disrupt the inner membrane (e.g., freeze–thaw cycles or the addition of chloroform or a mild detergent such as 0.1% Triton® X-100) due to the action of the protein on the outer wall when the inner membrane is disrupted.

TABLE II

Features of the BL21-Derived Competent Cells[a]

Expression strain	Features	Induction	Advantages	Disadvantages
BL21(DE3)pLysS competent cells[b]	General protein expression strain lacking both the *ompT* and *lon* proteases	IPTG induction of T7 RNA polymerase	Ease of induction	Slight inhibition of induced expression when compared with BL21(DE3) competent cells
	Encodes T7 RNA polymerase under the control of the *lacUV5* promoter			
	Contains the pLysS plasmid, a p15A derivative compatible with pET vectors and all ColE1 derivatives		Greater repression of T7 RNA polymerase	
	The pLysS plasmid codes for T7 lysozyme, a natural inhibitor of T7 RNA polymerase			
BL21(DE3) competent cells[b]	General protein expression strain lacking both the *ompT* and *lon* proteases	IPTG induction of T7 RNA polymerase from the *lacUV5* promoter	High level of expression and ease of induction	Leaky expression of T7 RNA polymerase can lead to uninduced expression of potentially toxic proteins
	Encodes T7 RNA polymerase under the control of the *lacUV5* promoter			
BL21 competent cells[b]	General protein expression strain lacking both the *ompT* and *lon* proteases	Infection with lambda bacteriophage CE6	Tight control of uninduced expression	Induction is not as efficient as DE3 derivatives
	Used with lambda CE6 for induction of protein synthesis under the control of T7 RNA polymerase			Induction (infection) process is more cumbersome

[a] See reference 7

BACTERIOPHAGE CE6

In cases in which target genes are too toxic to allow plasmids to be established in DE3 lysogens, T7 RNA polymerase can be delivered to the cell by infection with the bacteriophage CE6 by using the methods outlined in the Lambda CE6 Induction Kit (Catalog #235200), which is compatible with the CBP affinity-tag expression vectors. By using the method employed by the Lambda CE6 Induction Kit, no T7 RNA polymerase is present in the cell until the desired time of induction. The bacteriophage CE6 expresses T7 RNA polymerase from the lambda p_L and p_I promoters and carries the *Sam7* lysis mutations. This bacteriophage will allow effective expression of target genes in BL21 cells and presumably other nonrestricting hosts that absorb lambda. The phage can be propagated in the LE392 host strain [e14- (McrA-) *hsdR514 supE44 supF58 lacYI*],[13] which suppresses the *Sam7* mutation and therefore allows lysis of infected cells.

PREPARING THE VECTORS

- Perform a complete DNA digestion with the appropriate enzymes. Use *Nco* I and *Bam*H I for the pCAL-c vector, carefully ensuring that the proper coding sequence of the insert is in frame with the C-terminal tag. If the inserts to be cloned into these vectors contain one or more internal *Nco* I or *BamH* I sites, PCR primers may be engineered to include restriction sites with overhangs compatible with Nco I (e.g., *Afl* III, *Bsp*H I, *Sty* I) or *Bam*H I (e.g., *Bgl* II, *Bcl* I, *Bst*Y I).

- Any of the sites in the MCS can be used for the pCAL-n, pCAL-n-EK, and pCAL-n-FLAG vectors; however, ensure that the proper coding sequence of the insert is in frame with the N-terminal tag (see the MCS regions in Figures 2–4).

- Stratagene suggests dephosphorylation of the digested Affinity protein expression vector with CIAP prior to ligating to the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the DNA on an agarose gel and gel purifying the desired vector band eliminating the small fragment excised from between the two restriction enzyme sites.

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

LIGATING THE INSERT

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

$$\text{Picomole ends / microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 660}$$

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

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

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

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

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

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

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

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

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

TRANSFORMING THE CLONING REACTIONS

Following subcloning into the XL1-Blue strain, positive transformants are then used to transform a protein expression strain such as BL21-Gold (DE3).

Transformation Guidelines

It is important to store the competent cells at –80°C to prevent a loss of efficiency. For best results, please use the guidelines outlined in the following sections.

Storage Conditions

The competent cells are very sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. The competent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep the competent cells on ice at all times. It is essential that the Falcon® 2059 polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at least 100 µl of competent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of Falcon® 2059 Polypropylene Tubes

The use of Falcon 2059 polypropylene tubes when transforming into BL21-Gold (DE3) cells is imperative as the critical heat-pulsing period is calculated for the thickness and shape (i.e., the round bottom) of these tubes.

Quantity of DNA Added

Greatest efficiencies are observed when adding 1 µl of 0.1 ng/µl of DNA/100 µl of cells. A greater number of colonies will be obtained when plating up to 50 ng, although the overall efficiency may be lower.

Length of the Heat Pulse

Optimal transformation efficiencies are observed when transformation reactions are heat-pulsed for 20–25 seconds. Transformation efficiencies decrease sharply when the duration of the heat pulse is <20 seconds or >25 seconds.

TRANSFORMATION PROTOCOL

1. Thaw the BL21-Gold (DE3) competent cells on ice.

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

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

3. Add 1–50 ng of DNA to each transformation reaction and swirl gently. For the control transformation reaction, add 1 µl of the pUC18 control plasmid to a separate 100-µl aliquot of the competent cells and swirl gently.

4. Incubate the reactions on ice for 30 minutes.

5. Heat-pulse each transformation reaction in a 42°C water bath for 20 seconds. **The duration of the heat pulse is critical for optimal transformation efficiencies (see *Length of the Heat Pulse*).**

6. Incubate the reactions on ice for 2 minutes.

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

8. Concentrate the cells transformed with the ligation reaction by centrifugation and plate the entire transformation reaction (using a sterile spreader) onto a single LB agar plate[§] that contains the appropriate antibiotic.[‖]

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

9. Incubate the plates overnight at 37°C.

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

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

Expected Transformation Results

Host strain	Quantity of transformation plated	Expected colony number	Efficiency (cfu/μg of pUC18 DNA)
BL21-Gold(DE3)	5 μl	>50	$\geq 1 \times 10^8$

INDUCTION OF TARGET PROTEIN USING IPTG

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

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

1. Inoculate 1-ml aliquots of LB broth containing 100 μg/ml of carbenicillin or ampicillin (see *Preparation of Media and Reagents*) with single colonies from the transformation. Shake at 220–250 rpm at 37°C overnight.

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

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

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

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

   Note *These values for IPTG concentration and induction time are starting values only and may require optimization for the expression of different gene products.*

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

6. Pipet 20 µl of each of the induced cultures into clean microcentrifuge tubes. Add 20 µl of 2× SDS gel sample buffer (see *Preparation of Media and Reagents*) to each.

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

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

Induction of β-Galactosidase-CBP Fusion Protein Expressed from the pTC12 Vector

The pTC12 vector is included as a positive control for induction of CBP fusion protein. This vector contains the coding sequence for the *E. coli* β-galactosidase protein inserted between the *Nco* I and *Bam*H I sites of the pCAL-c vector. Induction of cultures of any of the BL21(DE3)-derived strains harboring the pTC12 plasmid should give rise to a prominent band of ~120 kDa when whole cell lysates are analyzed by SDS-PAGE.

The β-galactosidase-CBP fusion protein is completely insoluble when cultures are induced at 37°C. To purify the fusion, grow cultures at room temperature and induce for 5–10 hours at room temperature. Approximately 60–70% of the protein is soluble at 25°C.

PREPARING PROTEIN EXTRACTS

The method of extract preparation may vary depending on the physical characteristics of the protein and the preferences of the user. Any conventional lysis buffer may be used, provided $CaCl_2$ is included prior to the resin-binding step. For a list of buffer components that are compatible with CaM affinity resin, refer to *Table III.* **All steps are carried out at 4°C**.

Note *The following protocol is appropriate for a 1-liter volume of induced cell culture. The volumes given in the protocol can be scaled up or down to accommodate culture volumes other than 1 liter. Pellet the induced cells by centrifugation and proceed as directed below.*

1. Resuspend and pool the cell pellets in 30 ml of $CaCl_2$ binding buffer (see *Preparation of Media and Reagents*).
2. Add lysozyme to the cell suspension to a final concentration of 200 µg/ml and mechanically rotate the tube for 15 minutes.
3. Sonicate the sample for 30 seconds with the microtip at an intermediate setting. Cool the sample on ice for 3–5 minutes and repeat the sonicating–cooling cycle 4 more times.
4. Spin the sample for 15 minutes at high speed in a centrifuge and transfer the supernatant to a fresh tube for CaM affinity purification. Store the pellet at –80°C for further analysis if insolubility of the desired fusion protein is determined to be a concern.

TABLE III

Reagents Compatible with the Calmodulin Affinity Resin

Reagents	Comments
Sodium chloride (NaCl)	Use of high salt (1 M) may result in variable degrees of product loss. Sodium chloride (50–300 mM) has been found to be effective in the reduction of nonspecific interactions during binding, washing, and elution
Potassium chloride (KCl)	Same as above for NaCl
Dithiothreitol (DTT)[a]	Up to 5 mM may be used
β-Mercaptoethanol	Up to 10 mM may be used
Ammonium sulfate (NH_3SO_4)	Same as above for NaCl
Nonidet P-40 (NP-40)	Up to 0.1% (v/v)
1% Triton X–100	Up to 0.1% (v/v)
Protease inhibitors (2 µg/ml leupeptin, 2 µg/ml pepstatin, and 1 mM benzamidine)	Protease inhibitors, such as leupeptin, pepstatin, and benzamidine, are routinely used. The metal-ion-chelating agents, EGTA and EDTA, should be avoided, since these agents are used only during the elution process
Imidazole	Use 1 mM (typically)

PREPARING THE CALMODULIN AFFINITY RESIN

The calmodulin affinity resin is supplied in a storage buffer containing 20% (v/v) ethanol, 0.1 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.5), and 500 mM NaCl. Before the calmodulin affinity resin is used, the resin must be equilibrated to match the buffer constituents of the selected binding buffer. To prepare the calmodulin affinity resin, perform the following steps.

1. Decant the storage ethanol from the settled calmodulin affinity resin. Resuspend the resin in 5 bed volumes of the $CaCl_2$ binding buffer. Allow the slurry to settle.

2. Decant the supernatant again from the calmodulin affinity resin. Resuspend the resin in 5 bed volumes of the $CaCl_2$ binding buffer.

3. To complete the equilibration, again allow the calmodulin affinity resin to settle, decant the supernatant, and add an equal volume of the $CaCl_2$ binding buffer.

The calmodulin affinity resin is now ready for use in column packing (see *Standard Column Method*) or for use in batch binding (see *Batch Binding Method*). For small-scale purification of CBP fusion protein (50–150 µg), see *Small-Scale Quick Batch Method.*

PURIFYING THE PROTEIN

The efficiency of the extraction process depends on the amount of calmodulin affinity resin added to the crude lysate sample and on the level of expression of the desired product by *E. coli.* For recombinant fusion proteins, Stratagene typically obtains from 1.5 to 3.0 mg of pure protein/ml of resin added, depending on the size, and, to some extent, the conformation and physicochemical characteristics of the target protein to be purified. Visualizing the level of expression of the target protein using protein gels allows the user to conveniently estimate the correct amount of calmodulin affinity resin to add to a crude lysate sample.

Purification of β-Galactosidase-CBP Fusion Protein Expressed from the pTC12 Vector

After incubation of the extract with CaM affinity resin and extensive washing with calcium-containing binding buffer, wash the resin with 5–10 bed volumes of EGTA elution buffer (see *Preparation of Media and Reagents*) containing 150 mM NaCl, until there is no detectable A_{280}-absorbing material remaining in the wash fractions. Elute the purified fusion protein with EGTA elution buffer containing 1M NaCl. Recovery of ~16 mg of soluble fusion protein can be expected from 1 liter of culture.

Standard Column Method

Note *Before proceeding, Stratagene recommends reviewing the* Batch Binding Method *and the* Small-Scale Quick Batch Method *immediately following this section to determine the preferred method of protein purification. Protein purification may be carried out at 4°C or room temperature.*

1. Degas the equilibrated calmodulin affinity resin.

   Note *Care should be taken in the following step to ensure that all the material components of the column, including the calmodulin affinity resin, are the same temperature in order to avoid bubble formation in the packed column.*

2. Fill the appropriate size column with a 10% volume of $CaCl_2$ binding buffer to eliminate air pockets.
3. Pour the degassed calmodulin affinity resin into the column by running the resin down the shaft of a glass rod. This procedure will prevent air bubble formation in the packing process. Immediately fill 80% of the remaining column space with $CaCl_2$ binding buffer and affix a clamped column adapter (filled with $CaCl_2$ binding buffer) in order to meet the top of the fluid in the column. Connect the column to a pump.
4. Open the bottom of the column and set the pump to run at the desired rate to create a bed of resin. When the packing is complete, turn off the pump and lower the adapter to meet the top of the resin bed.
5. The column is now ready to load with the crude lysate sample.

6. After loading the sample, wash the column with 5–10 column volumes of binding buffer to remove unbound material. [More stringent washing procedures may be employed if necessary. (See *Troubleshooting* for more information.)]

7. Proteins are subsequently released (eluted) from the column matrix by removal of the calcium from the calmodulin affinity resin. In the absence of calcium, calmodulin undergoes a conformational change, releasing the affinity-tagged fusion protein. Calcium removal is preferably achieved by chelation with EGTA. EDTA at 2 mM in an elution buffer may also be used [50 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 2 mM EDTA, and 150 mM NaCl]. Many variations of elution buffer are possible. Refer to Table III for more information.

   Note *While most proteins elute efficiently with buffers containing 2 mM EGTA and low salt, many proteins require an additional elution with 50 mM Tris-HCl (pH 8.0), 2 mM EGTA, and 1 M NaCl to recover immobilized fusion proteins.*

Batch Binding Method

1. Add the equilibrated calmodulin affinity resin directly to the crude lysate sample and allow the sample to interact with the resin and to bind from several hours to overnight at 4°C with mechanical rotation.

2. After binding, pour the slurry into a column and generate a resin bed.

   Note *Save the material that flows through the column for subsequent verification that the desired product has been effectively removed from the extract.*

3. Wash the column using at least 10 column volumes of binding buffer to remove unbound material. Prior to elution, verify that there is no A_{280}-absorbing material in the final calcium-containing washes. This may be determined using standard Coomassie-based protein determination reagents.

4. Elute the product with 10 column volumes of elution buffer. Many variations of elution buffer are possible. Refer to Table III for more information.

   Note *While most proteins elute efficiently with buffers containing 2 mM EGTA and low salt, many proteins require an additional elution with 50 mM Tris-HCl (pH 8.0), 2 mM EGTA, and 1 M NaCl to recover immobilized fusion proteins.*

Small-Scale Quick Batch Method

Small-scale purification of CBP fusion proteins (50–150 µg) can be done in a microcentrifuge tube. This method is also useful for optimizing conditions for larger-scale purifications.

1. Aliquot ~50 µl of resin into a 1.5-ml microcentrifuge tube and pellet the beads by spinning at low speed for 2 minutes at 1000 rpm in a microcentrifuge. Equilibrate the calmodulin affinity resin with $CaCl_2$ binding buffer by performing four 200-µl washes with $CaCl_2$ binding buffer following each resuspension of the pellet with a low-speed spin.

2. Resuspend the equilibrated calmodulin affinity resin with the crude *E. coli* lysate and bring the slurry to a total volume of ≥300 µl with binding buffer. Mechanically rotate the tube at 4°C for 2 hours.

3. Pellet the beads and remove the unbound material. Save this fraction for further analysis.

4. Wash the beads 4–6 times with 300 µl of binding buffer. The final wash fraction should contain no detectable protein by SDS–PAGE or a negligible amount of protein by spectrophotometric protein determination assays (see *Troubleshooting* for variations in the washing regimen).

5. Elute the fusion protein by four or more sequential washes with 200 µl of elution buffer until the fractions no longer contain detectable levels of purified fusion proteins as assessed by protein determination assays or SDS–PAGE.

   Note *While most proteins elute efficiently with buffers containing 2 mM EGTA and low salt, many proteins require an additional elution with 50 mM Tris-HCl (pH 8.0), 2 mM EGTA, and 1 M NaCl to recover immobilized fusion proteins.*

REGENERATING THE CALMODULIN AFFINITY RESIN

1. Wash the calmodulin affinity resin with 3 column volumes of 0.1 M $NaHCO_3$ (pH 8.6) containing 2 mM EGTA.
2. Wash with 3 column volumes of 1 M NaCl containing 2 mM $CaCl_2$.
3. Wash with 3 column volumes of 0.1 M acetate buffer (pH 4.4) containing 2 mM $CaCl_2$.
4. Wash with binding buffer containing 1–2 mM $CaCl_2$.

Notes *Stratagene does not recommend regenerating the calmodulin affinity resin more than three times.*

Denatured proteins or lipids that do not elute in the regeneration procedure can be removed by washing the resin with a 0.1% nonionic detergent (e.g., Triton X-100) at 37°C for 1 minute followed by re-equilibration with binding buffer.

Store the regenerated CaM affinity resin at 4°C in 20% (v/v) ethanol.

REMOVING THE CBP PURIFICATION TAG WITH THROMBIN

1. Dialyze or dilute the CBP fusion protein into thrombin cleavage buffer (see *Preparation of Media and Reagents*). Add the thrombin to the reaction tube and incubate at room temperature until cleavage is complete.

   Note *If EGTA-containing fractions are diluted directly into the protease cleavage buffer, Stratagene recommends adding a compensatory amount of $CaCl_2$, so the final effective $CaCl_2$ concentration is 2.5 mM.*

2. Determine the efficiency of proteolytic removal of the CBP affinity tag by SDS–PAGE analysis. Ideal digestion conditions will vary between proteins and should be optimized for each fusion protein. Stratagene recommends starting with a 1:500 thrombin-to-fusion protein ratio and analyzing the reaction products at various time points from several minutes to 24 hours following the addition of thrombin. A lower thrombin-to-target ratio (e.g., 1:50) may be used to decrease long reaction times.

   Note *Thrombin may be inactivated by the addition of 0.5 mM PMSF.*

3. Uncleaved fusion protein and free CBP can be absorbed by incubation with calmodulin affinity resin in the presence of 2mM $CaCl_2$ and $\geq$200mM NaCl. The resin is removed from the sample by low-speed centrifugation (~1000 rpm in a standard microcentrifuge).

REMOVING THE CBP PURIFICATION TAG WITH ENTEROKINASE

The pCAL-n-EK vector and the pCAL-n-FLAG vector contain an EK recognition site. The following protocol may be used to cleave the fusion protein and subsequently remove the cleavage product.

Cleaving the Fusion Protein

1. Dialyze or dilute the purified CBP fusion protein into EK cleavage buffer (see *Preparation of Media and Reagents*).

2. Add one unit of EK for every 100 µg of fusion protein to be cleaved and incubate the reaction for up to 24 hours at room temperature until cleavage is complete. Assess the cleavage efficiency by SDS–PAGE.

Removing the Enterokinase and Cleavage Product

1. Adjust the NaCl concentration of the reaction to 200 mM.

2. Add a mixed slurry of calmodulin affinity resin and STI–agarose to the digest. **The resins should be mixed and extensively washed with EK buffer containing 200mM NaCl before addition to the EK reaction mix.**

Note *1.0 ml of CaM affinity resin will bind 2 mg of CBP-fusion protein. To remove enterokinase, add 10uL of STI-agarose per every unit of enterokinase. However, it is recommended that not less than 10 µl of either resin be used in reactions containing small quantities of protein.*

3. Mechanically rotate at 4°C for 30 minutes and then remove the resin by low-speed centrifugation (~1000 rpm in a standard microcentrifuge) for 2–3 minutes.

TROUBLESHOOTING

Observations	Suggestions
Plasmid instability	Unstable DNA sequence. Prior to induction of cultures, assay for colony formation by plating cells on an LB plate and an LB-ampicillin plate. If the plasmid contains unstable DNA sequence one should observe colony formation on the LB plate, and reduced colony formation on the LB-ampicillin plate
	Overexpression of toxic proteins. Prior to induction of cultures, assay for colony formation by plating cells on an LB-ampicillin plate and an LB-ampicillin plate containing IPTG. If the insert codes for a protein that is toxic to the cells, overexpression of the toxic protein should result in reduced colony formation on an LB-ampicillin plate containing IPTG as compared to cells plated on the LB-ampicillin plate
	More tightly controlled induction may be achieved by performing induction by infecting BL21 cells with the bacteriophage CE6
Problems associated with induction time	Depends on the physicochemical characteristics of the protein and toxicity of the protein to *E. coli*. In certain cases, accumulation of target protein may kill cells at saturation while allowing normal growth in logarithmically growing cultures, while in other cases target protein may continue to accumulate in cells well beyond the recommended 3-hour induction period. To determine the optimal induction period, a time course may be carried out during which a small portion of the culture is analyzed by SDS–PAGE at various times following induction
Inclusion bodies	Improper folding in *E. coli* and/or bacterial aggregation due to the physical properties of the protein. In some cases, protein may form insoluble inclusion bodies at 37°C. In many cases, this protein may be soluble and active if the induction is carried out at 30°C. Inclusion body formation may be used as a purification step by simply spinning out the insoluble material from crude lysates and redissolving the protein in urea or guanidinium-HCl
The fusion protein fails to bind to the calmodulin affinity resin	Insufficient $CaCl_2$ in the binding buffer due to omission or chelation (e.g., due to presence in extract of agents, such as EDTA or EGTA). Increase the amount of $CaCl_2$ in the binding buffer from the range of 0.2–2 mM $CaCl_2$
	Affinity tag is inaccessible due to conformation of fusion protein, which rarely occurs. Stratagene recommends recloning the protein-coding sequence of interest with the tag at the opposite terminus

Observations	Suggestions
Fusion protein observed in calcium-containing residual flow-through and wash fractions	The capacity of the calmodulin affinity resin for a specific fusion protein will vary slightly, depending on the size and physical characteristics of the protein. When planning a purification, Stratagene recommends using 1.0 ml of calmodulin affinity resin for every 2.0 mg of fusion protein estimated present in the extract. Estimates of protein expression can be made by visual analysis via stained SDS–PAGE gels or by probing electroblots with a protein-specific antibody or with biotinylated calmodulin (bio-CaM) using the *Affinity CBP Fusion Protein Detection Kit*
	The amount of resin to use per unit volume of extract has not been optimized. A more rigorous method for optimizing the amount of resin to use per unit volume of extract is to set up a series of binding reactions using the *Small-Scale Quick Batch Method* of protein purification. Using this method, increasing volumes of extract are incubated with a fixed amount of resin, and the flow-through fractions are then analyzed by probing electroblots with specific antibody or with bio-CaM
	The largest volume of extract added to the resin for which there is no detectable fusion protein in the $CaCl_2$ flow-through can be used to determine the minimal amount of resin required to deplete the extract of fusion protein in a scaled up purification (the Affinity CBP fusion protein detection kit can detect ≥10 ng of fusion protein in crude extract)
Precipitation of fusion protein observed in elution fractions	Insufficient ionic strength in the elution buffer and/or the pH of the elution buffer is inappropriate for the pH of the fusion protein. Optimize the buffer system to correct the ionic strength in the elution buffer or correct the pH of the elution buffer affecting the pH of the fusion protein. Stratagene recommends using the *Small-Scale Quick Batch Method* of protein purification
Contaminating proteins coeluting with fusion protein	Proteolytic degradation of fusion protein. This protein degradation will result in bands of reduced molecular weight, which can be visualized by SDS–PAGE and may be verified by probing electroblots with antibody or bio-CaM. Inclusion of various commercially available protease inhibitors in the binding, wash, and elution buffers can be highly effective in reducing this degradation
	Copurification of contaminating proteins has occurred. Increase the ionic strength of the binding and wash buffer up to 300 mM NaCl. This step is frequently effective in the reduction of undesirable ionic interactions. In extreme cases, the use of up to 1 M NaCl may be required to obtain pure protein, although a coincidental reduction in yield may result due to nonspecific elution of some fusion protein
	Alternatively, the use of nonionic detergents, such as NP-40 and Triton X-100, at 0.1% may be effective in the elimination of contaminating proteins. These detergents may be applied in conjunction with the variable salt conditions described under troubleshooting observation "*The fusion protein fails to bind to the calmodulin affinity resin*"
"Bleeding" of fusion protein over several fractions during elution when using the *Batch Binding Method* of protein purification	A typical elution profile exhibits ~70% of the purified protein in the first 2–4 fractions, with a trailing edge extending out. For the yield-conscious user, a concentration step is commonly employed on this trailing edge prior to storage

Observations	Suggestions
Protein fails to elute completely from the resin	In some cases, lower than anticipated yields may occur in the elution steps. Protein yields can be verified by boiling a small portion of the resin in Laemmli sample buffer and analyzing by SDS–PAGE. Stratagene finds that washing the column with 3-column volumes of buffer containing 2 mM EGTA and 1 M NaCl is often effective for eluting tightly bound proteins
	Alternatively, fusion protein bound tightly to the resin has been eluted successfully using buffers of low-ionic strength containing 2 mM EGTA (in this case, the tight binding is presumably due to nonspecific hydrophobic interactions between the CBP affinity tag and the calmodulin affinity resin)
	Finally, in some cases, denatured proteins or proteins associated with lipids do not elute efficiently. The use of nonionic detergents in conjunction with EGTA has been found to be effective for some proteins in these situations
Incomplete proteolytic cleavage	The efficiency of proteolytic removal of the CBP affinity tag will vary from protein to protein, and in some cases, the conformation of the protein may inhibit accessibility of the thrombin- or EK cleavage target site for the enzyme. Longer incubation times or higher concentrations of protease may help
	Positioning of the tag at the opposite terminus of the protein of interest by recloning the insert into the appropriate Affinity protein expression vector may increase accessibility of the target site

PREPARATION OF MEDIA AND REAGENTS

CIAP Buffer (10×)
500 mM Tris-HCl (pH 8.0)
1 mM EDTA

$CaCl_2$ Binding Buffer
50 mM Tris-HCl (pH 8.0)
150 mM NaCl
10 mM β-mercaptoethanol
1.0 mM magnesium acetate
1.0 mM imidazole
2 mM $CaCl_2$

Elution Buffer
50 mM Tris-HCl (pH 8.0)
10 mM β-mercaptoethanol
2 mM EGTA
150 mM NaCl

Enterokinase Cleavage Buffer
50 mM Tris-HCl (pH 8.0)
50 mM NaCl
2 mM CaCl2
0.1% Tween-20

Laemmli Sample Buffer (2×)
250 mM Tris HCl (pH 6.8)
8% (w/v) sodium dodecyl sulfate (SDS)
40% (v/v) glycerol
0.01% (w/v) bromophenol blue dye
700 mM β-mercaptoethanol

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

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

LB–Ampicillin Broth (per Liter)
1 liter of LB broth, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin

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

LB–Carbenicillin Broth (per Liter)
Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 10 ml of 10-mg/ml-filter-sterilized carbenicillin

Ligase Buffer (10×) 500 mM Tris-HCl (pH 7.5) 70 mM $MgCl_2$ 10 mM dithiothreitol (DTT) ***Note*** *rATP is added separately in the ligation reaction*	**2× SDS gel sample buffer** 100 mM Tris-HCl (pH 6.5) 4% SDS (electrophoresis grade) 0.2% bromophenol blue 20% glycerol ***Note*** Add dithiothreitol to a final concentration in the 2× buffer of 200 mM prior to use. **This sample buffer is useful for denaturing, discontinuous acrylamide gel systems only.**
SOB Medium (per Liter) 20.0 g of tryptone 5.0 g of yeast extract 0.5 g of NaCl Add deionized H_2O to a final volume of 1 liter Autoclave Add 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$ prior to use Filter sterilize	**SOC Medium (per 100 ml)** SOB medium Add 1 ml of a 2 M filter-sterilized glucose solution or 2 ml of 20% (w/v) glucose prior to use Filter sterilize
TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA	**Thrombin Cleavage Buffer** 20 mM Tris-HCl (pH 8.4) 150 mM NaCl 2.5 mM $CaCl_2$

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ENDNOTES

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