Affinity® LIC Cloning and Protein Purification Kit

INSTRUCTION MANUAL

Catalog #214405 (Affinity® LIC Cloning and Protein Purification Kit) and
#214407 (Affinity® LIC Cloning Kit)
Revision #121004a

For In Vitro Use Only

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Stratagene. Stratagene shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

United States and Canada

Stratagene
11011 North Torrey Pines Road
La Jolla, CA 92037
Telephone (858) 535-5400
Order Toll Free (800) 424-5444
Technical Services (800) 894-1304
Internet tech_services@stratagene.com
World Wide Web www.stratagene.com

Stratagene European Contacts

Location	Telephone	Fax	Technical Services
Austria	0800 312 526	0800 312 527	017 956 7036
Belgium	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 96078	0800 96024	027 13 12 11
France	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 100391	0800 881323	
Germany	00800 7000 7000	00800 7001 7001	00800 7400 7400
	06221 400 634	06221 400 639	06221 400 637
	0130 840 911	0130 762 088	069 9509 6197
Netherlands	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 023 0446	0800 023 0447	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	061 6930 540	061 6930 544	01 800 9045
	0800 830 250	0800 825 225	
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	01223 420 955	01223 420 234	0171 365 1056
	0800 585 370	0800 783 0889	

All Other Countries

Please contact your local distributor. A complete list of distributors is available at *www.stratagene.com*.

Affinity® LIC Cloning and Protein Purification Kit

CONTENTS

Affinity® LIC Cloning and Protein Purification Kit

MATERIALS PROVIDED

Material provided	Quantity	
	Catalog #214405	**Catalog #214407**
pCAL-n-FLAG vector (20 ng/µl)[a]	400 ng of DNA	400 ng of DNA
Test insert (kanamycin gene, 1.17 Kb, 30 ng/µl)	3 µl	3 µl
pTC12 control plasmid (1 µg/µl)	10 µg	10 µg
SoloPack® Gold supercompetent cells [b]	21 transformations	21 transformations
pUC18 control plasmid (0.1 ng/µl)	2× 10 µl	2× 10 µl
XL10-Gold β-Mercaptoethanol	50 µl	50 µl
Cloned *Pfu* DNA polymerase (0.5 U/µl) [c]	55 µl	55 µl
10× Cloned Pfu polymerase buffer	30 µl	30 µl
10 mM dATP[c]	30 µl	30 µl
BL21-Gold(DE3) competent cells [d]	5 × 0.2-ml aliquots	—
Enterokinase (EK)	50 U	—
STI-agarose	250 µl	—
Calmodulin (CaM) affinity resin [e]	5 ml	—
EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (100 mM)]	1 ml	—

[a] Cesium chloride-banded, predigested plasmid DNA. Sufficient vector is provided for 20 total cloning reactions. The complete sequence and list of restriction sites for the pCAL-n-FLAG vector are available at *www.stratagene.com*. The pCAL-n-FLAG vector sequence is also available from the GenBank® database (Accession #AF087042).

[b] Efficiencies are ≥1 × 10^9 transformants/µg.

[c] The cloned *Pfu* DNA polymerase provided is formulated specifically for this kit; successful PCR results will not be achieved using this formulation.

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

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

STORAGE CONDITIONS

SoloPack® Gold Supercompetent Cells: –80°C
pUC 18 (Control Plasmid): –80°C
XL10-Gold β-Mercaptoethanol: –80°C
BL21-Gold (DE3) Competent Cells: –80°C
Enterokinase: –80°C
pCAL-n-FLAG Vector: –20°C
Test Insert: –20°C
pTC12 Control Plasmid: –20°C
Cloned *Pfu* DNA Polymerase: –20°C
10× Cloned *Pfu* DNA Polymerase Buffer: –20°C
10 mM dATP: –20°C
EGTA: –20°C
Calmodulin Affinity Resin: 4°C
STI-agarose: 4°C

Revision #121004a

ADDITIONAL MATERIALS REQUIRED

Kanamycin
Chloramphenicol
Isopropyl-1-thio-β-D-galactopyranoside (IPTG)
Lysozyme
Falcon® 2059 polypropylene tubes (15-ml)
Disposable columns and glass rods for pouring the degassed resin
Deoxynucleoside triphosphate
Pfu DNA polymerase†
Taq DNA polymerase†
pET 3´ 18-mer primer (5´ CTAGTTATTGCTCAGCGG 3´)
pCAL-n 5´ 21-mer primer (5´ GAATTTCATAGCCGTCTCAGC 3´)
Gel purification system
Sonicator

† See *Endnotes.*

NOTICES TO PURCHASER

FLAG® License Agreement

The enclosed DNA expression vector and/or antibody are specifically adapted for a method of producing selected protein molecules covered by one or more of the following patents owned by Sigma-Aldrich Co.: U.S. Patent Nos. 4,703,004, 4,782,137 and 4,851,341. Your payment includes a limited license under these patents to make only the following uses of these products:

A. **Vector License:** You may use the enclosed vector to transform cells to produce proteins containing the amino acid sequence DYKDDDDK for research purposes provided, however, such research purposes do not include binding an unlicensed antibody to any portion of this amino acid sequence nor using such proteins for the preparation of antibodies having an affinity for any portion of this amino acid sequence.

B. **Antibody License:** You may only use the enclosed antibody for research purposes to perform the method of producing proteins covered by claims 5 and 6 of U.S. Patent No. 4,703,004 so long as: (1) you perform such method with a DNA expression vector licensed from Sigma-Aldrich Co.; and (2) you do not bind (or allow others to bind) an unlicensed antibody to any DYKDDDDK epitope of any fusion protein that is produced by use of the method.

This license does not include any rights under any other patents. You are not licensed to use the vector and/or antibody in any manner or for any purpose not recited above. As used above, the term "unlicensed antibody" means any antibody which Sigma-Aldrich Co. has not expressly licensed under claims 5 and 6 of U.S. Patent No. 4,703,004. Sigma-Aldrich Co. hereby expressly retains all rights in the above listed patents not expressly licensed hereunder.

If the terms and conditions of this License Agreement are acceptable to you, then you may open the vessel(s) containing the vector and/or antibody and, through such act of opening a vessel, will have shown your acceptance to these terms and conditions.

If the terms and conditions of this License Agreement are not acceptable to you, then please return the vessel(s) unopened to Stratagene for a complete refund of your payment.

For additional licensing information or to receive a copy of any U.S. Patent Nos. 4,703,004, 4,782,137 or 4,851,341, please contact the Sigma-Aldrich Co. licensing department at telephone number 314-771-5765.

Academic and Nonprofit Laboratory Assurance Letter

The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is protected by U.S. patents assigned to Brookhaven Science Associates (BSA). BSA will grant a nonexclusive license for use of this technology, including the enclosed materials, based on the following assurances:

1. These materials are to be used for noncommercial research purposes only. A separate license is required for any commercial use, including the use of these materials for research purposes or production purposes by any commercial entity. Information about commercial licenses may be obtained from the Office of Intellectual Property and Industrial Partnerships, Brookhaven National Laboratory, Bldg. 475D, Upton, New York, 11973 [telephone (631) 344-7134].

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.

You may refuse this license by returning the enclosed materials unused. By keeping or using the enclosed materials, you agree to be bound by the terms of this license.

Commercial Entities Outside of the US

The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is protected by U.S. Patents assigned to Brookhaven Science Associates (BSA). To protect its patent properties BSA requires commercial entities doing business in the United States, its Territories or Possessions to obtain a license to practice the technology. This applies for in-house research use of the T7 system as well as commercial manufacturing using the system. Commercial entities outside the U.S. that are doing business in the U.S., must also obtain a license in advance of purchasing T7 products. Commercial entities outside the U.S. that are using the T7 system solely for in-house research need not obtain a license if they do no business in the United States. However all customers, whether in the U.S. or outside the U.S. must agree to the terms and conditions in the Assurance Letter which accompanies the T7 products. Specifically, 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 the assurance letter 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.

To obtain information about licensing, please contact the Office of Intellectual Property and Industrial Partnerships, Brookhaven National Laboratory, Building 475D, Upton, NY 11973 [telephone: 631-344-7134; Fax: 631-344-3729].

INTRODUCTION

The Affinity® LIC cloning and protein purification kit is designed for high-level production and one-step purification of seamless calmodulin-binding peptide (CBP) fusion proteins from *Escherichia coli* (*E. coli*) extracts. The pCAL-n-FLAG® vector, derived from the pCAL-n-EK vector,[1,2] allows fusion of the CBP purification tag followed by the FLAG epitope, to the N-terminus of the protein-coding sequence of interest. The FLAG epitope has been added to allow for an antibody recognition site on a protein of interest for which the CBP tag would be problematic. Protein synthesis is tightly repressed in the absence of inducer. Upon induction, CBP–FLAG fusion proteins are consistently produced at high levels due in part to the exceptional efficiency of translation of the CBP fusion tag when positioned at the N terminus of the fusion. CBP fusion proteins can be purified from crude cell extracts to near homogeneity with one pass through calmodulin (CaM) affinity resin using moderate buffer conditions.

The Affinity LIC cloning and protein purification kit uses ligation-independent cloning (LIC)[3,4] to obtain high-efficiency cloning of the desired coding sequence into the pCAL-n-FLAG vector. LIC creates seamless cloning junctions between the protein-coding sequence of interest and the recognition target for the site-specific protease enterokinase (EK). The EK target sequence [$(Asp)_4$-Lys] is located between the FLAG epitope and the N terminus of the protein of interest. Because EK cleaves at the C terminus of its recognition target, cleavage of fusion proteins generated from the pCAL-n-FLAG vector yields the recovery of C-terminal fusion partners free of extra amino acids derived from the fusion tag (see Figure 1). A modified LIC cloning method allows the positioning of any protein-coding sequence immediately C-terminal to the EK cleavage site, without any sequence constraints imposed by the cloning method. Following purification of the fusion protein and proteolytic removal of the CBP–FLAG tag, the desired protein can be efficiently recovered from reactions containing EK, free CBP–FLAG, and, in some cases, uncleaved fusion protein, with a mixed resin slurry of CaM affinity resin and STI-agarose. (STI-agarose binds specifically to the catalytic site of EK.)

The CBP affinity tag is based on the high affinity ($K_d = 10^{-9}$) for the protein CaM exhibited by a 26-amino-acid C-terminal fragment from muscle myosin light-chain kinase at physiological pH in the presence of calcium.[5–7] When calcium is removed from the environment, CaM undergoes a conformational change that results in the release of its ligand (see Figure 2). CBP fusion proteins produced from pCAL-n-FLAG bind to immobilized CaM with high affinity in a wide range of calcium-containing buffers. The small size of the 4-kDa CBP affinity tag allows production of soluble target proteins of a larger size than those that are commonly achieved with glutathione *S*-transferase (GST) (26 kDa) or maltose-binding protein (MBP) (40 kDa). The CBP affinity tag is also less likely to affect the function of the protein of interest than the GST and MBP purification tags. In addition to its use as an affinity tag for protein purification, a target sequence for protein kinase A (PKA) within the CBP allows the fusion protein to be efficiently radiolabeled with PKA in the presence of [γ-^{32}P]ATP. Thus, high-specific-activity probes can be generated for interaction cloning or for blot overlay detection of interacting proteins.[8]

In some instances, the researcher may wish to use a calmodulin affinity purified protein to study protein:protein interactions in immunoprecipitation "pull-down" experiments using eukaryotic cell extracts. The FLAG epitope is well-characterized for use in immunoprecipitation.[2] The CBP tag is undesirable for use in such applications in that the presence of endogenous calmodulin binding proteins, which are ubiquitous in all eukaryotic cell types, may contribute to background. A FLAG fusion protein free of the CBP purification tag can be generated by thrombin cleavage. Free CBP and any uncleaved protein is easily removed with calmodulin affinity resin.

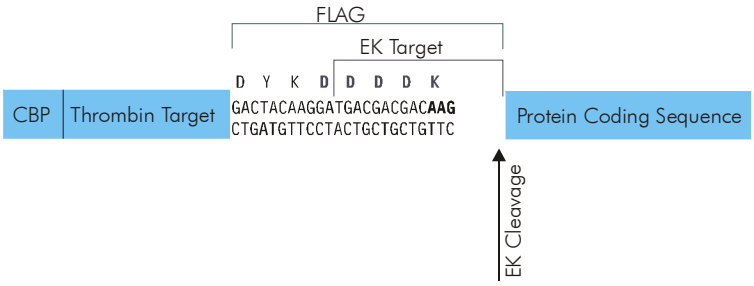


FIGURE 1 Predicted n-terminal amino acid sequence of pCAL-n-FLAG fusion proteins. The vector is designed to allow seamless fusion of protein-coding sequence of interest to the C terminus of the FLAG affinity tag followed by the EK cleavage site. Following one-step affinity purification of the CBP–FLAG fusion protein using calmodulin affinity resin, it may be cleaved with enterokinase to yield recombinant protein of native amino acid sequence, or thrombin to yield FLAG fusion protein free of the CBP purification tag.

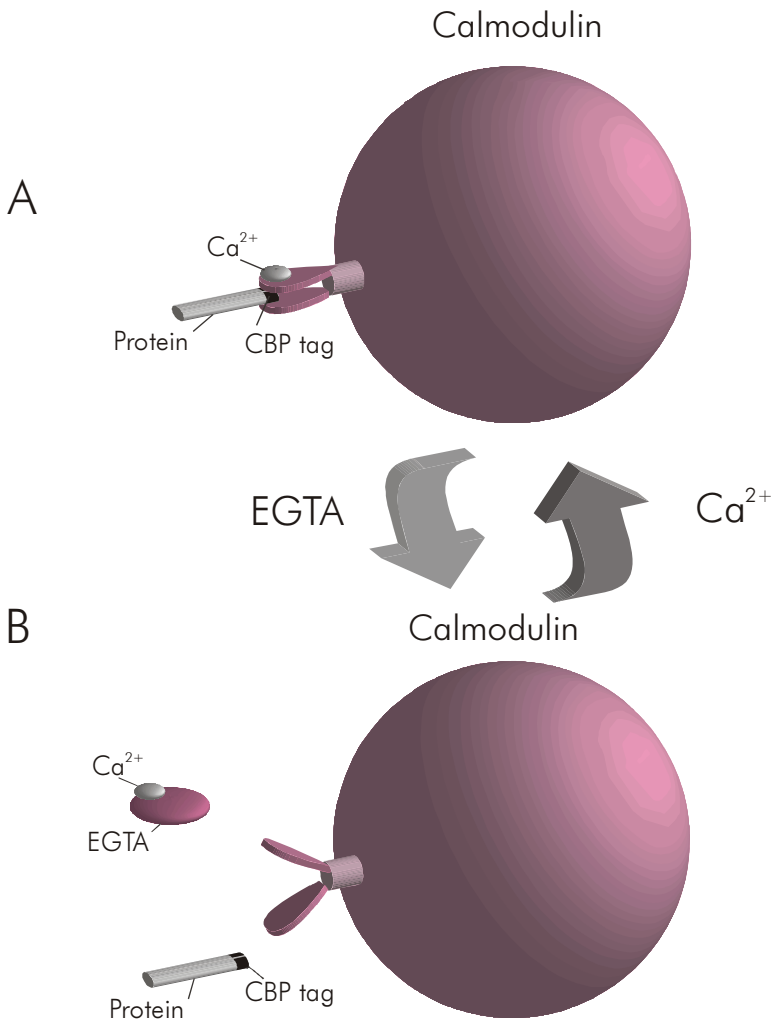


FIGURE 2 The Affinity protein expression and purification system. The highly conserved protein calmodulin binds to the CBP-tagged fusion protein in the presence of low concentrations of calcium at neutral pH (A). The fusion protein elutes from its ligand at neutral pH with 2 mM EGTA (B). The purified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

THE pCAL-n-FLAG VECTOR

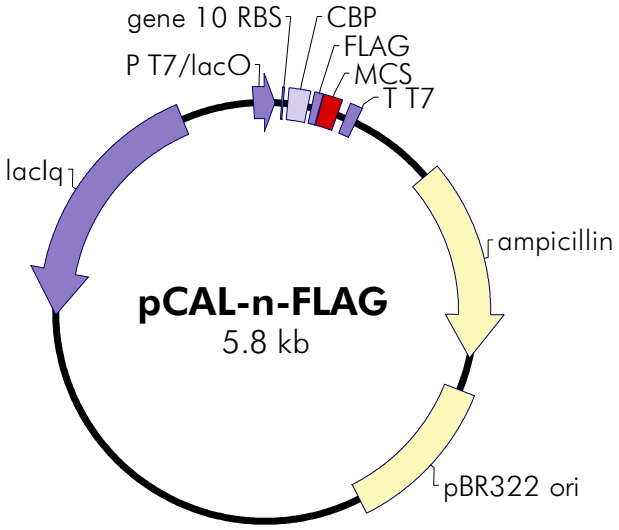
The pCAL-n-FLAG vector (see Figure 3) is derived from the pET-11 vector series, which was engineered to take advantage of the features of the bacteriophage T7 gene *10* promoter and leader sequence. These T7-derived features allow high selectivity of the promoter by T7 RNA polymerase and tight repression. The pCAL-n-FLAG vector uses the T7 *lac* promoter configuration and carries a copy of the *lacI*^{q} gene to mediate this tight repression. The vector is provided LIC-ready, prepared with 12- and 13-nt single-stranded overhangs (see Figure 4). The pTC12 vector is included as a positive control for induction and purification of CBP fusion proteins.

The pCAL-n-FLAG vector encodes the five-amino-acid recognition target for the site-specific protease enterokinase (EK), positioned between the FLAG epitope and the cloning site. The cloning site in pCAL-n-FLAG is engineered such that digestion, followed by treatment with *Pfu* DNA polymerase† in the presence of dTTP, yields 12- and 13-nt single-stranded overhangs. The overhangs are annealed to similarly-prepared PCR fragments, allowing the ligation-independent insertion of any desired sequence immediately 3′ to the fifth codon (AAG) of the EK recognition site (see Figure 4). The CBP affinity tag allows for rapid one-column purification of the expressed protein and isotopic labeling of purified protein.

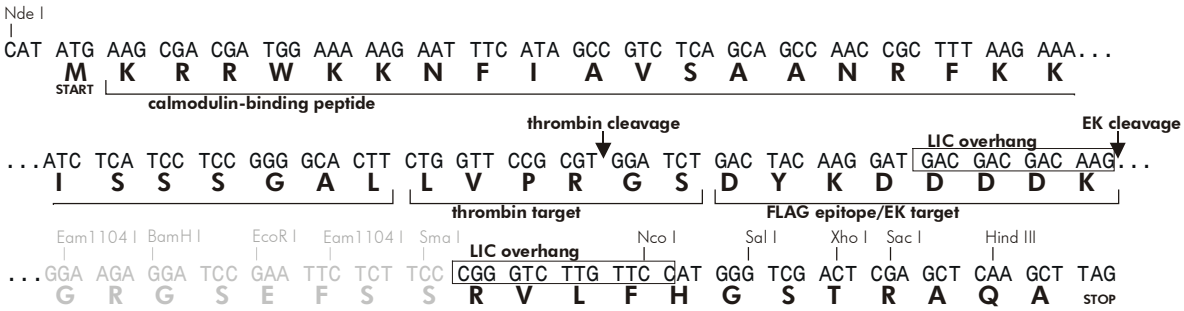
Vector Primers and Coordinates

Vector	Primer	Coordinates	Sequence
pCAL-n-FLAG	pCAL-n 5′ 21-mer	109–129	5′ GAATTTCATAGCCGTCTCAGC 3′
	pET 3′ 18-mer	323–340	5′ CTAGTTATTGCTCAGCGG 3′

The pCAL-n-FLAG Vector Circular Map and Features



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



Note *The pCAL-n-FLAG vector provided with this kit has been LIC-adapted, with the boxed sequences present as ss-DNA overhangs. The sequence shown in gray is absent from the LIC-adapted vector. The position numbers listed below correspond to the pCAL-n-FLAG vector prior to LIC adaptation.*

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	4360–5448

FIGURE 3 The pCAL-n-FLAG vector

LIC Cloning with pCAL-n-FLAG

The LIC method used to clone the protein-coding sequence of interest into the pCAL-n-FLAG vector allows the fusion of any protein-coding sequence immediately C-terminal to the EK cleavage site. The upstream cloning junction (i.e., N-terminal with respect to the protein-coding sequence of interest) contains a 5´, twelve-nucleotide single-stranded (ss) overhang in the antisense strand whose 5´ end corresponds to the anticodon for the C-terminal lysine in the EK recognition site. Similarly, the downstream cloning junction contains a 5´, thirteen-nucleotide ss overhang in the coding strand.

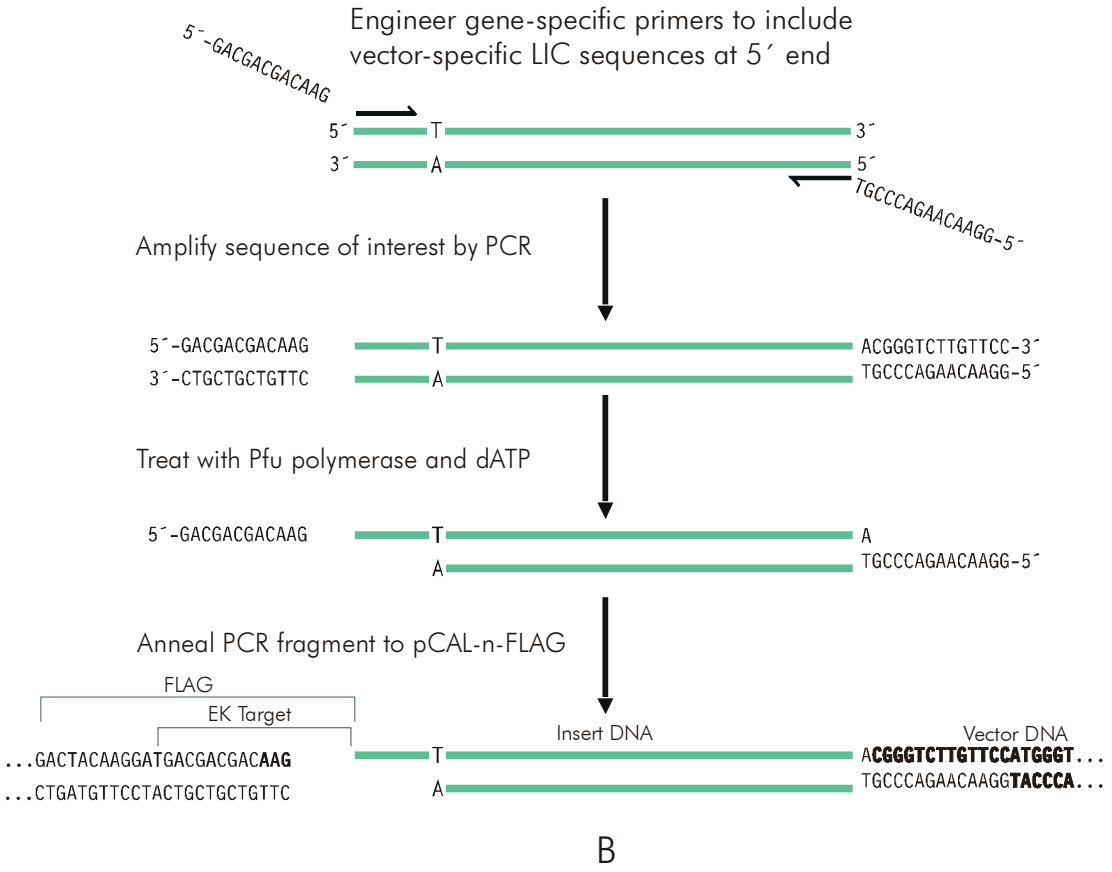
To generate PCR inserts, primers are designed as shown in Figure 4, with the vector-specific 12- and 13-nt sequences added to the 5´ end of the insert-specific sequences. For the upstream primer, the insert-specific codon located immediately 3´ to the vector-specific Lys codon at the 3´ end of the EK recognition sequence should correspond to the desired N-terminal amino acid in the mature, EK cleavage product. Because the multiple cloning site (MCS) does not contain a stop codon, the downstream primer should be designed so that the insert-specific sequence either contains an in-frame stop codon or hybridizes to a sequence downstream of the stop codon.

Following PCR amplification, the PCR product is treated with *Pfu* polymerase in the presence of dATP. The 3´–5´ exonuclease activity of *Pfu* polymerase removes nucleotide residues from both 3´ ends of the PCR product to generate 5´ ss overhangs that are complimentary to those in the vector. The exonucleolytic removal of nucleotide residues will continue until the first dAMP residue is encountered in the strand. This dAMP residue is engineered into the downstream primer. In the upstream primer, however, the dAMP residue will correspond to the first dAMP that naturally occurs in the antisense strand of the insert.

Following *Pfu* treatment, the insert is annealed to the vector without any further enzymatic treatment and the annealed product is used to transform SoloPack® Gold supercompetent cells. LIC efficiencies of $>10^5$ cfu/µg have been routinely obtained using the protocol provided in this instruction manual.

...GACTACAAGGAT CGGGTCTTGTTCCATGGGTCGACTC...
...CTGATGTTCCTACTGCTGCTGTTC TACCCAGCTGAG...

A



B

FIGURE 4 Ligation-independent cloning of the protein-coding sequence to produce seamless CBP–FLAG fusion proteins. (A) The pCAL-n-FLAG vector preparation provided with the kit has the single-stranded, 12- and 13-nt overhangs shown in the diagram and requires no further manipulation. (B) Insert preparation: PCR primers are engineered to include the 5′ sequence shown in the diagram. The gene-specific sequence engineered immediately 3′ to the vector-specific sequence for the sense strand primer should correspond to the desired N-terminal amino acid codons, which will be fused immediately C-terminal to the EK cleavage site. Following treatment with *Pfu* DNA polymerase in the presence of dATP, the PCR fragment is annealed to the single-stranded overhangs of the pCAL-n-FLAG vector, and the annealed DNA is then used to transform SoloPack Gold supercompetent cells without the need for ligation or any further enzymatic manipulation. Note that the 3′ end of the antisense strand will be digested in the 3′ to 5′ direction until the first adenine base (A) in the inserted sequence of interest is reached, potentially resulting in a single-stranded gapped duplex following annealing of the insert to the vector. This gap is repaired *in vivo* following transformation of the annealed DNA.

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.[9–12] 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[9,10] 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.[13] 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, however, SoloPack® Gold supercompetent cells are recommended and provided for even higher efficiency in LIC cloning.

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., SoloPack Gold supercompetent cells) for cloning and then transforming BL21-Gold(DE3)pLysS cells with miniprep DNA for expression.

Host Strain Genotypes

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.

The pCAL-n-FLAG vector uses the T7 *lac* promoter configuration and contains a copy of the *lacI* gene. For optimal CE6 induction of this vector, derepression of the plasmid-borne expression cassette with IPTG following infection is recommended and should be optimized for each gene.

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]

TABLE I

Features of the BL21-Derived Competent Cells[a]

Expression strain	Features	Induction	Advantages	Disadvantages
BL21(DE3)pLysS competent cells	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	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	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 14

BACTERIOPHAGE CE6

In cases in which target gene products 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, 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 which absorb lambda. The phage can be propagated in the LE392 host strain [e14$^-$ (McrA$^-$) *hsdR514 supE44 supF58 lacYI*],[15] which suppresses the *Sam7* mutation and therefore allows lysis of infected cells.

PREPARING THE VECTOR

The pCAL-n-FLAG vector DNA provided in this kit is restriction-digested and pretreated with *Pfu* DNA polymerase in the presence of dTTP. The 3´-to-5´ exonuclease activity of *Pfu* removes nucleotides until it reaches the first dTMP in the vector sequence, revealing 12- and 13-nt single-stranded overhangs. This allows seamless LIC of appropriately prepared insert DNA. The pCAL-n-FLAG vector requires no further manipulation (see Figure 4 and *Ligation-Independent Cloning*).

LIGATION-INDEPENDENT CLONING

1. Design PCR primers that contain a pCAL-n-FLAG-specific sequence followed by an insert-specific sequence as outlined below:

   - 5´ PCR primer: 5´ GAC GAC GAC AAG [X_{21} (insert-specific)] 3´ (sense)
   - 3´ PCR primer: 5´ GGA ACA AGA CCC GT [X_{21} (insert-specific)] 3´ (antisense)

2. Amplify the desired experimental insert by PCR using appropriate primers under conditions optimized for the experimental insert. To minimize mutation frequency, the number of PCR cycles can be reduced to between 10 and 20 due to the small quantity of insert required for the reaction.

3. Gel purify the experimental insert from any extraneous bands and PCR components.

4. Calculate picomole ends/µg DNA as follows:

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

5. Prepare the following reactions in thin-walled PCR tubes:

LIC Control Reaction	Experimental Reaction
1 µl of kanamycin test insert	10–100 fmol of purified experimental insert
1 U of cloned *Pfu* DNA polymerase	1 U of cloned *Pfu* DNA polymerase
1 µl of 10× cloned *Pfu* DNA polymerase reaction buffer	1 µl of 10× cloned *Pfu* DNA polymerase reaction buffer
1 µl of 10 mM dATP	1 µl of 10 mM dATP
dH_2O to a final volume of 10 µl	dH_2O to a final volume of 10 µl

6. Incubate the reactions at 72°C for 15 minutes and then place on ice for 2 minutes.

7. Add 1 µl (20 ng) of the provided pCAL-n-FLAG vector to each reaction.

8. Incubate the reactions at least one hour at room temperature to allow the insert to anneal to the pCAL-n-FLAG single-stranded overhangs. (The reactions may be incubated overnight at room temperature. Overnight annealing may increase the cloning efficiency.)

9. Transform **0.75** µl of the **LIC control reaction** into one aliquot of SoloPack Gold supercompetent cells and transform **2** µl of the **experimental reaction** into one aliquot of SoloPack Gold supercompetent cells as outlined in *SoloPack Gold Transformation*.

10. Plate the experimental transformants on LB–ampicillin plates. Plate the control transformants on LB-kanamycin plates. Resultant transformed colonies can be screened for the desired experimental insert by colony PCR using the gene-specific primers designed in step 1 or by using vector-specific primers.

    Note *Plating 100 µl of the LIC control transformation on LB–kanamycin (50 µg/ml) agar plates should yield >50 colonies. This yield can increase to >100 colonies with overnight annealing.*

11. Transform positive clones into BL21-Gold (DE3) competent cells (see *BL21-Gold (DE3) Transformation*) for use in *Induction of Target Protein Using IPTG*.

TRANSFORMING THE LIC REACTIONS AND THE RESULTANT POSITIVE CLONES

The LIC reactions are transformed into SoloPack supercompetent cells. Following subcloning, positive transformants are then used to transform a protein expression strain such as BL21(DE3) (see *BL21(DE3) Expression Strain*).

Transformation Guidelines

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 the BL21-Gold (DE3) 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 β-Mercaptoethanol with SoloPack Supercompetent Cells

β-Mercaptoethanol (β-ME) has been shown to increase the transformation efficiency. The XL10-Gold® β-mercaptoethanol mix provided in this kit is diluted and ready for use. For optimum efficiency, use 1 µl of the β-mercaptoethanol mix. (Stratagene cannot guarantee the highest efficiency with β-ME from other sources.)

Use of Falcon 2059 Polypropylene Tubes with BL21-Gold (DE3) Cells

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 to 50 µl of cells. A greater number of colonies will be obtained when plating up to 50 ng, although the overall efficiency may be lower.

Duration of the Heat Pulse for BL21-Gold (DE3) Cells

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.

Temperature and Duration of the Heat Pulse for SoloPack Supercompetent Cells

The temperature and duration of the heat pulse (54°C for 60 seconds) have been optimized to obtain the highest transformation efficiency in the supplied tube. Different temperatures, durations, and tubes result in decreased transformation efficiency.

SOLOPACK GOLD TRANSFORMATION

1. Thaw 3 tubes of SoloPack Gold cells on ice (one tube for the LIC control reaction, one tube for the experimental reaction, and one tube for the transformation control).

2. When the cells have thawed, swirl the tubes to gently mix the cells.

3. Add 1 µl of the XL10-Gold β-mercaptoethanol mix provided in the kit to each tube. (Stratagene cannot guarantee the highest efficiency with β-mercaptoethanol from other sources. See *Use of β-Mercaptoethanol with SoloPack Supercompetent Cells*.)

4. Swirl the tubes gently. Incubate the cells on ice for 10 minutes, swirling the tubes every 2 minutes.

5. Add 2 µl of the experimental LIC reaction to one tube of cells and add 0.75 µl of the LIC control reaction to a second tube of cells. Swirl gently. As a transformation control, add 1 µl of the pUC18 DNA control plasmid (diluted 1:10 in high-quality water) to the third tube of cells and swirl gently.

6. Incubate the tubes on ice for 30 minutes.

7. Preheat NZY+ broth (see *Preparation of Media and Reagents*) in a 42°C water bath for use in step 10.

   Notes *Transformation of SoloPack Gold cells has been optimized with **NZY+ broth**.*

   Preheating the NZY+ broth is optional. It is not essential for achieving the highest transformation efficiency.

8. Heat-pulse the tubes of SoloPack Gold supercompetent cells in a **54°C** water bath for 60 seconds.

   Notes *The temperature and duration of the heat pulse are **critical** for obtaining the highest transformation efficiency (see* Temperature and Duration of the Heat Pulse for SoloPack Supercompetent Cells*).*

   For consistent results, remove any ice that may be trapped around the outside bottom of the tube.

9. Incubate the tubes on ice for 2 minutes.

10. To each tube of SoloPack Gold supercompetent cells, add 0.15 ml of 42°C NZY$^+$ broth and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.

11. Plate 200 µl (or less) of the experimental LIC transformation reaction on LB–ampicillin plates using a sterile spreader. Plate 200 µl (or less) of the LIC control transformation reaction on LB–kanamycin plates using a sterile spreader.

    To plate the cells transformed with the pUC18 DNA control plasmid, first place a 200-µl pool of NZY$^+$ broth on an LB–ampicillin agar plate (see *Preparation of Media and Reagents*), then add 5 µl of the transformation control reaction to the pool. Use a sterile spreader to spread the mixture.

12. Incubate the plates overnight at 37°C.

Expected Results

Transformation reaction	Quantity of transformation plated	Expected colony number	Efficiency (cfu/µg of pUC18 DNA)
pUC18 DNA	5 µl	250	≥1 × 10^9
LIC control reaction	100 µl	>50	—

BL21-GOLD (DE3) TRANSFORMATION

1. Thaw the competent cells on ice.

   Note *Store the competent cells **on ice at all times** while aliquoting. It is essential that the Falcon 2059 polypropylene tubes are placed on ice before the competent cells are thawed and that 100 µl of competent cells are aliquoted directly into each **prechilled** polypropylene tube. Pipet the remaining competent cells into 100-µl aliquots and freeze the aliquots at –80°C. 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. Preheat SOC medium[§] in a 42°C water bath for use in step 8.

6. Heat-pulse each transformation reaction in a 42°C water bath for 20 seconds. **The duration of the heat pulse is critical for optimal transformation efficiencies (*see Duration of the Heat Pulse for BL21-Gold (DE3) Cells*).**

7. Incubate the reactions on ice for 2 minutes.

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

9. Concentrate the transformation reactions by centrifugation and plate the entire 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.

10. Incubate the plates overnight at 37°C.

Expected Results

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

[§] 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.

INDUCTION OF TARGET PROTEIN USING IPTG

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

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

1. Inoculate 1-ml aliquots of LB broth (see *Preparation of Media and Reagents*) containing the antibiotic required to maintain the expression plasmid with single colonies from the transformation. Shake at 220–250 rpm at 37°C overnight.

   Note *For BL21-Gold(DE3)pLysS host strain, the overnight culture must contain chloramphenicol at a final concentration of 50 µg/ml in addition to the antibiotic required to maintain the expression plasmid. Chloramphenicol serves to maintain the pACYC-based plasmid carrying T7 lysozyme gene derivative.*

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 depending on the gene expressed.*

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

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

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

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.[14]

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 THE PROTEIN EXTRACT

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 II*. **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 II

Reagents Compatible to 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)	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	Commonly use 1 mM

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 (see *Troubleshooting* for more information).

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 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 *To avoid bubble formation in the packed column, ensure all components of the column, including the calmodulin affinity resin, are the same temperature.*

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 used if necessary (See *Troubleshooting*). Prior to elution, verify that there is no A_{280} absorbing material in the final calcium-containing washes. This may also be determined using standard Coomassie-based protein determination reagents.

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. Many variations of elution buffer are possible. Refer to Table II 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 II 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 with 3 column volumes of 0.1 M $NaHCO_3$ (pH 8.6) with 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 sodium acetate buffer (pH to 4.4 with acetic acid) containing 2 mM $CaCl_2$.

4. Wash with binding buffer containing 1–2 mM $CaCl_2$.

Notes *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 reequilibration with binding buffer.*

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

REMOVING THE CBP–FLAG FUSIONS WITH ENTEROKINASE

The pCAL-n-FLAG vector contains an EK recognition site at the C-terminus of the FLAG epitope. 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–FLAG 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 CBP–FLAG

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 or STI-agarose will bind 2 mg of their respective ligands; 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).

REMOVING THE CBP PURIFICATION TAG WITH THROMBIN

The pCAL-n-FLAG vector contains a thrombin cleavage site between the CBP and FLAG fusions that facilitates the removal of the CBP purification tag from a FLAG fusion protein.

1. Dialyze or dilute the CBP–FLAG 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).

TROUBLESHOOTING

Observations	Suggestions
Low colony yield after transformation of the LIC reaction into SoloPack cells	dNTPs are not removed from the PCR reaction resulting in interference with the LIC reaction. Remove all dNTPs from the PCR reaction using gel or column purification Quantitate DNA concentration after gel purification of insert to ensure excess insert DNA not preventing proper exonucleolytic activity.
Plasmid instability	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 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 20–30°C (longer incubation periods should be used for lower temperatures). 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. Alternatively, linker peptides inserted between the CBP and protein of interest may improve the accessibility of the purification tag. Stratagene recommends the peptide $(Gly_4Ser)_3$
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*

(*table continues on the next page*)

(*table continued from the previous page*)

Observations	**Suggestions**
Fusion protein is observed in calcium-containing residual flow-through and wash fractions	Optimize 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	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 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
Contaminating proteins coeluting with fusion protein	Copurification of contaminating proteins has occurred. Increase the ionic strength of the binding and wash buffer up to 300 mM NaCl. This 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
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

(*table continues on the next page*)

(*table continued from the previous page*)

Observations	**Suggestions**
Protein fails to elute completely from the resin	In some cases, lower than anticipated yields may occur in the elution steps. 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) 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–FLAG and CBP affinity tags will vary from protein to protein, and in some cases, the conformation of the protein may inhibit accessibility of the cleavage target site for EK or thrombin. Longer incubation times or higher protease concentrations may be helpful. The efficacy of cleavage can be monitored by SDS–PAGE analysis of time points from the cleavage reaction. For large fusion proteins for which the mobility shift is not easily ascertained by gel staining, anti-FLAG western blot or the CBP-fusion protein detection test may be used to determine the presence or absence of the tags. Positioning of the tag at the opposite terminus of the protein of interest by recloning the insert into the appropriate protein expression vector may increase accessibility of the target site

PREPARATION OF MEDIA AND REAGENTS

$CaCl_2$ 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) bromphenol blue dye
700 mM β-mercaptoethanol

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 Agar (per Liter)
Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

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–Ampicillin Broth (per Liter)
Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized ampicillin

LB–Carbenicillin Broth (per Liter)
Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized carbenicillin

LB–Kanamycin Agar (per Liter)
Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

NZY+ Broth (per Liter)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
Autoclave
Add the following supplements before use
12.5 ml of 1 M $MgCl_2$
12.5 ml of 1 M $MgSO_4$
10 ml of 2 M filter-sterilized glucose solution **or** 20 ml of 20% (w/v) glucose
Filter sterilize

Thrombin Cleavage Buffer

20 mM Tris-HCl (pH 8.4)
150 mM NaCl
2.5 mM $CaCl_2$

SOC Medium (per 100 ml)

Note *Prepare immediately before use*

1 ml of a 2 M filter-sterilized glucose solution **or** 2 ml of 20% (w/v) glucose
SOB medium to a final volume of 100 ml
Filter sterilize

REFERENCES

1. Wyborski, D. L., Bauer, J. C., McGowan, B., Sorge, J. and Vaillancourt, P. (1997) *Strategies* 10(1):15–18.
2. Felts, K., Wyborski, D. L., Bauer, J. C. and Vaillancourt, P. (1999) *Strategies* 12(1):24–25.
3. Aslanidis, C. and de Jong, P. J. (1990) *Nucleic Acids Res* 18(20):6069–74.
4. Haun, R. S., Serventi, I. M. and Moss, J. (1992) *Biotechniques* 13(4):515–8.
5. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D., Bishop, S. M., Acott, T. S. *et al.* (1991) *J Biol Chem* 266(22):14188–92.
6. Stofko-Hahn, R. E., Carr, D. W. and Scott, J. D. (1992) *FEBS Lett* 302(3):274–8.
7. Means, A. R., Bagchi, I. C., Van Berkum, M. F. and Rassmussen, C. D. (1991). In *Cellular Calcium: A Practical Approach*, J. G. McCormack and P. H. Cobbold (Eds.). IRL Press, Oxford, England.
8. Blanar, M. A. and Rutter, W. J. (1992) *Science* 256(5059):1014–8.
9. Studier, F. W. and Moffatt, B. A. (1986) *J Mol Biol* 189(1):113–30.
10. Weiner, M. P., Anderson, C., Jerpseth, B., Wells, S., Johnson-Browne, B. *et al.* (1994) *Strategies* 7(2):41–43.
11. Phillips, T. A., VanBogelen, R. A. and Neidhardt, F. C. (1984) *J Bacteriol* 159(1):283–7.
12. Grodberg, J. and Dunn, J. J. (1988) *J Bacteriol* 170(3):1245–53.
13. Jerpseth, B., Callahan, M. and Greener, A. (1997) *Strategies* 10(2):37–38.
14. Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) *Methods Enzymol* 185:60–89.
15. Borck, K., Beggs, J. D., Brammar, W. J., Hopkins, A. S. and Murray, N. E. (1976) *Mol Gen Genet* 146(2):199–207.

ENDNOTES

† Purchase of these products is accompanied by a license to use them in the Polymerase Chain Reaction (PCR) process in conjunction with an Authorized Thermal Cycler. Stratagene's PCR products are sold under licensing arrangements with Roche Molecular Systems, Inc., F. Hoffmann-La Roche Ltd., and The Perkin-Elmer Corporation.

†† Practice of the patented Polymerase Chain Reaction (PCR) process requires a license. Stratagene's thermal cycler is an Authorized Thermal Cycler. Its use with Authorized Reagents provides a limited PCR license in accordance with the label rights accompanying such reagents. It may also be used with PCR licenses available from The Perkin-Elmer Corporation.