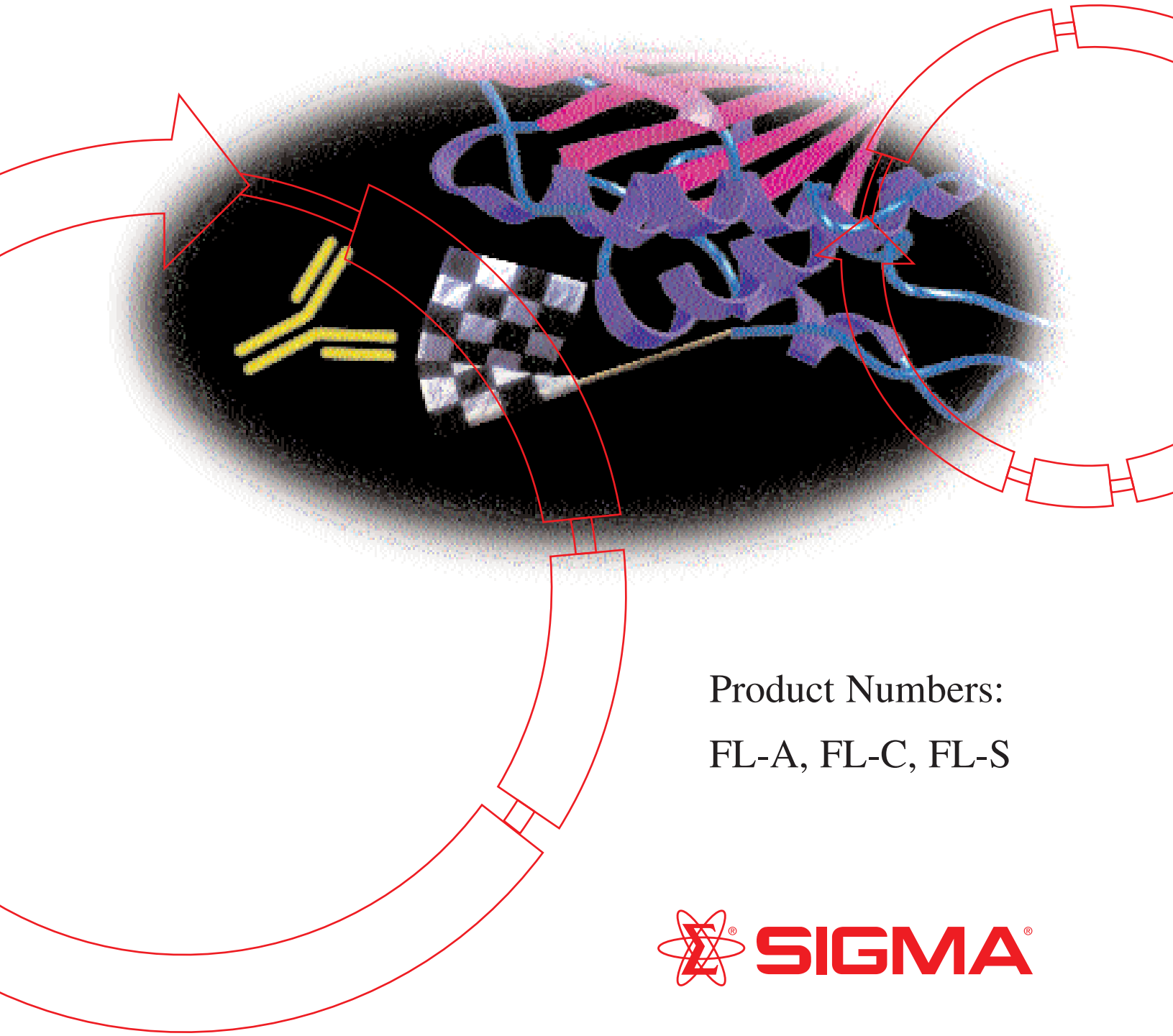
E. coli Expression System Manual



Product Numbers:

FL-A, FL-C, FL-S

SIGMA®

INTRODUCTION

General Description

The FLAG expression system[1] is based on the FLAG marker octapeptide **(Figure 1).** The FLAG peptide is fused to the protein of interest using a FLAG expression vector where the coding sequence of the FLAG peptide is adjacent to and either upstream or downstream from the multiple cloning site (MCS). The open reading frame (ORF) of the protein of interest is then cloned into the MCS. Upon translation, the expressed protein will be either an N-terminal or C-terminal fusion of the FLAG peptide and the protein of interest. The ANTI-FLAG® mouse monoclonal antibodies M1 (IgG2b) and M2 (IgG1) can specifically bind to the eight amino acid FLAG peptide for the detection of the FLAG fusion protein in a variety of immunological procedures. In addition, both the M1 and M2 ANTI-FLAG antibodies have been conjugated to a gel matrix, allowing for affinity chromatography to purify FLAG fusion proteins.

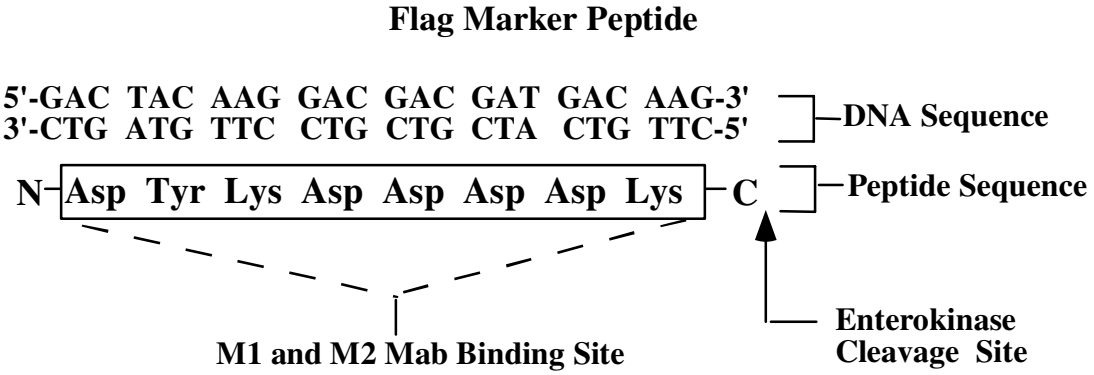


Figure 1. Sequence of the Flag Marker Peptide. All the eight amino acids are required for binding of the antibodies M1 and M2. Enterokinase recognizes the Asp-Asp-Asp-Asp-Lys sequence at the carboxy terminal and cleaves after Lys. Note that cleavage will not occur if Lys is followed by a Pro. In addition, Enterokinase cleavage (see page 35) does not occur at an internal location or at the carboxy terminus of the FLAG fusion protein. Also note that the FLAG DNA sequence may vary from one FLAG vector to the next, but the peptide sequence remains the same.

General Strategy for Expression

The *E. coli* FLAG Expression System has been designed for fusion of the FLAG peptide to proteins for expression in *E. coli*. The ORF is cloned into an appropriate vector to achieve the expression of the FLAG fusion protein. The general strategy for the expression of the fusion protein is given in **Figure 2**.

Figure 2.

General Outline of Methodology for Expression of a Fusion Protein in FLAG *E. coli* Expression System.

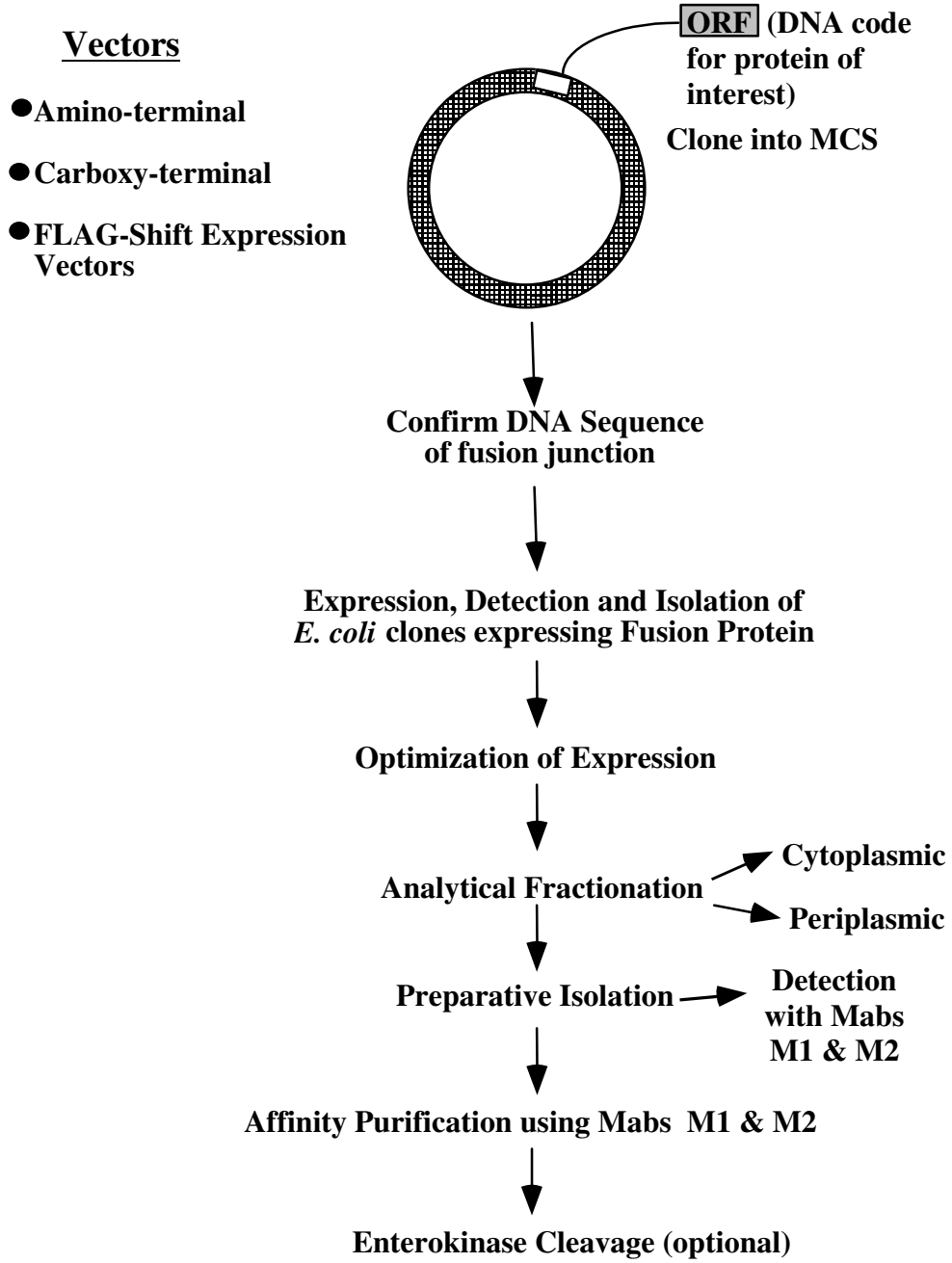


Table 1.

Recommended Components of the *E. coli* Expression System for the Purification and Detection of the FLAG Fusion Proteins

Expression Vector	Size (bp)	Control Plasmid	Control Protein	FLAG Fusion	Confirm Fusion	Localization	Protein Detection	Protein Purification	Enterokinase
pFLAG-MAC	5071	PFLAG-ATS -BAP	N-Terminal FLAG-BAP	Met	N-26	Cytoplasm	M2	M2 Gel	Yes
pFLAG-Shift$_{12c}$	5074	pFLAG-Shift $_{12}$ -BAP							
pFLAG-2	5052	pFLAG-1-BAP							
pFLAG-CTC	5336	pFLAG-CTS-BAP	C-Terminal FLAG-BAP	C	C-24	Cytoplasm	M2	M2 Gel	No
pFLAG-ATS	5396	pFLAG-ATS-BAP	N-Terminal FLAG-BAP	N	N-26	Periplasm	M1 M2	M1 Gel M2 Gel	Yes
pFLAG-Shift$_{12}$	5134	pFLAG-Shift $_{12}$ -BAP							
pFLAG-1	5377	pFLAG-1-BAP							
pFLAG-CTS	5391	pFLAG-CTS-BAP	C-Terminal FLAG-BAP	C	C-24	Periplasm	M2	M2 Gel	No

For detailed information on the various *E. coli* Expression Kits, refer to Tables 2, 3 and 4

Choice of Vector

The ORF can be cloned into an amino-terminal or carboxy-terminal FLAG Expression Vector if the phase of the reading frame is known. Expression is possible using the FLAG-Shift Expression Vectors if the amino-terminus need not or cannot be precisely defined and/or the reading frame is unknown. FLAG-Shift Expression Vectors contain a "shift" sequence that allows expression of an ORF without regard to the reading frame in which it was initially cloned. (See section on FLAG-Shift Expression Vectors). The FLAG-Shift Expression Vectors are especially useful for cloning an ORF from a genomic, shotgun or cDNA library when the reading frame of an ORF is not known.

All vectors share similar multiple cloning sites (MCS) which allow transfer of an ORF between each member of the FLAG Expression Vector family. This feature allows transfer of an ORF with little manipulation prior to cloning. Expression vectors offered in kits contain a MCS with restriction sites represented in each of the three reading frames. Restriction enzyme digestion of a site within each MCS can yield 5' overhangs, 3' overhangs or blunt ends. The pFLAG-1 and pFLAG-2 Expression Vectors, which are only available separately, share an identical MCS, with all restriction sites in the same reading frame. Restriction enzyme digestion of any site within the MCS (of FLAG-1 and FLAG-2) yields a four base, 5' overhang.

Confirmation of Sequence of Fusion Junction of ORF

The DNA sequence of potential FLAG clones must be verified at the FLAG fusion junction for proper phasing. While restriction analysis is necessary, it is usually not sufficient to confirm the exact sequence of the FLAG fusion junction.

The N-26 and C-24 oligodeoxyribonucleotide primers are provided for sequencing either the amino- or carboxy-terminal FLAG fusion junctions of the inserted gene sequence. The N-26 and C-24 primers are suitable for use in cycle sequencing. They may also be useful in amplification techniques for sequence modification of FLAG fusion proteins.

Expression of Fusion Protein: Cytoplasmic or Periplasmic

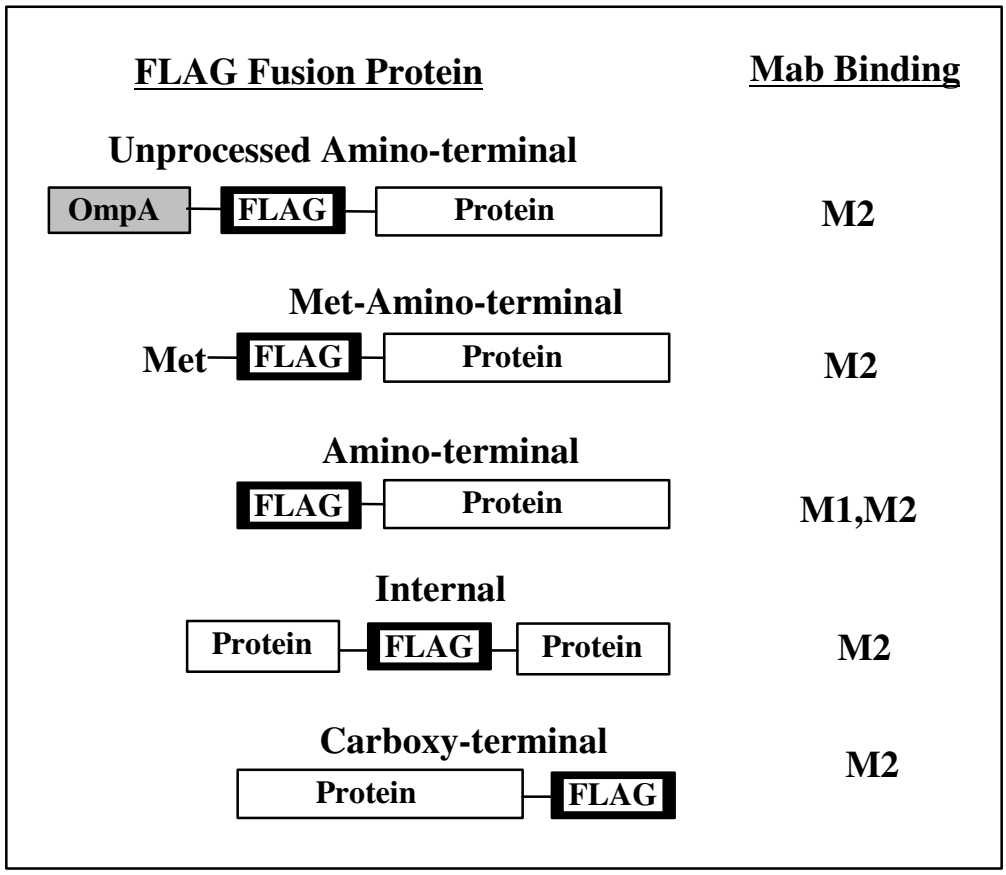
Each of the amino-terminal, carboxy-terminal or FLAG-Shift Expression Vectors is offered as pairs. Selection depends on whether cytoplasmic or periplasmic expression of a FLAG fusion protein is desired. One member of each pair encodes the OmpA secretion signal coding sequence for periplasmic expression of a FLAG fusion protein. The second member of each pair lacks the OmpA secretion signal coding sequence, which results in cytoplasmic expression of a FLAG fusion protein.

Purification and Detection of FLAG Fusion Protein

Expression of the FLAG fusion protein from an *E. coli* FLAG Expression Vector permits affinity purification with the ANTI-FLAG M1 or M2 Affinity Gel. The gel used depends on the location of the FLAG marker within the protein. **(Table 1.)** The ANTI-FLAG M1 and M2 Affinity gels allow purification of biologically active proteins using mild conditions. Mild elution conditions using the calcium-dependent ANTI-FLAG M1 Affinity Gel include chelation with EDTA and/or competitive elution with FLAG peptide. FLAG fusion proteins can be eluted from the ANTI-FLAG M2 Affinity Gel using peptide elution. FLAG fusion proteins can be detected by Western or dot blot with the ANTI-FLAG M1 Monoclonal Antibody if the FLAG marker is located at a free amino-terminus. The ANTI-FLAG M2 Monoclonal Antibody is used if the FLAG marker is at the amino or carboxy-terminus. The FLAG peptide provides a useful marker for further characterization of the FLAG fusion protein using a range of immunological techniques. However, it can be removed from amino-terminal or Met-amino-terminal fusion proteins using enterokinase. A guide to the binding of the Monoclonal Antibodies M1 and M2 to various fusion proteins is represented in **Figure 3.**

Figure 3.

Binding of Monoclonal Antibodies M1 & M2 to FLAG Fusion Proteins



Features and Applications of *E.coli* FLAG Expression System

The FLAG peptide has several features that make it useful for the affinity purification and immunological detection of FLAG fusion proteins:

- **Efficient:** Development of a specialized scheme including raising antibodies to the protein or development of a functional assay is not required to purify the protein.
- **Versatile:** Amino-terminal or carboxy-terminal FLAG fusion proteins can be expressed in *E. coli*, yeast, insect or animal cells (See Section below).
- **Minimal effect on protein function:** The small octapeptide has minimal effect on the conformation of the native protein.
- **Ease of detection:** The eight amino acid sequence has a high surface probability**.** A surface location maximizes its accessibility to the ANTI-FLAG M1 and M2 Monoclonal Antibodies.
- **Mild purification:** Rapid affinity purification of FLAG fusion proteins with the ANTI-FLAG M1 or M2 Affinity Gel employs mild conditions for recovery of a biologically active protein.

- **Ease of removal:** Contains the rare, five amino acid recognition sequence for enterokinase. This enables recovery of an intact protein following its proteolytic removal.
- **Multiple applications:** Useful for further study of protein-protein, protein-DNA interactions, protein surveillance and ultrastructure.

The FLAG technology has been used for expression of proteins in a variety of cell types including *E.coli,*[1-4, 11,13,14,17,18,20,24,45,49,51,59] yeast,[1,2,46] insect cells[22,47,60,61] and mammalian cells.[3-10,12,15,16,19,21,23,48,49] The proteins expressed have been used for a variety of purposes including receptor studies,[50-54, 56, 62] signal transduction[55, 63] and apoptosis[57,64].

E. coli FLAG EXPRESSION KITS

The following *E. coli* Expression kits are available:

1. ***E. coli* Amino-Terminal FLAG Expression kit (Table 2)**
2. ***E. coli* Carboxy-Terminal FLAG Expression kit (Table 3)**
3. ***E. coli* FLAG-Shift Expression Kit (Table 4)**

Table 2.

Components of the *E. coli* Amino-Terminal FLAG Expression Kit

Kit Component*	Function	Storage
pFLAG-ATS™ Expression Vector	Expression and secretion of Amino-Terminal FLAG Fusion protein in periplasm	0 to –20°C
pFLAG-MAC™ Expression Vector	Expression and secretion of Met-Amino-Terminal FLAG Fusion protein in cytoplasm	0 to –20°C
pFLAG-ATS-BAP™ Control Plasmid	Positive control for protein expression, detection and purification	0 to –20°C
N-24 Primer	Sequencing of Amino-Terminus of FLAG Fusion junction	0 to –20°C
C-24 Primer	Sequencing of Carboxy-Terminus of FLAG Fusion junction	
ANTI-FLAG M1 Monoclonal Antibody	Immunological detection of Amino-terminal FLAG fusion protein from pFLAG-ATS Expression Vector	0 to –20°C
ANTI-FLAG M2 Monoclonal Antibody	Immunological detection of Met Amino-terminal FLAG fusion protein from pFLAG-MAC Expression Vector and Amino-terminal FLAG fusion protein from pFLAG-ATS Expression Vector	
ANTI-FLAG M1 Affinity Gel	Affinity purification of Amino-terminal FLAG fusion protein from pFLAG-ATS Expression Vector	2 to 8°C
ANTI-FLAG M2 Affinity Gel	Affinity purification of Amino-terminal FLAG fusion protein from pFLAG-ATS Expression Vector or Met-Amino-terminal FLAG fusion protein from PFLAG-MAC Expression Vector	
FLAG Peptide	Peptide elution of FLAG fusion protein from ANTI-FLAG M1 or M2 Affinity Gel	2 to 8°C
N-Terminal FLAG-BAP Control Protein	Positive control for detection and purification	0 to –20°C
Enterokinase	Removal of FLAG peptide from Amino-terminal and Met-Amino-Terminal FLAG Fusion protein	0 to –20°C

*** See Footnotes to Table 4**

Table 3.

Components of the *E. coli* Carboxyl-Terminal FLAG Expression Kit

Kit Component*	Function	Storage
pFLAG-CTS Expression Vector	Expression and secretion of Carboxy-Terminal FLAG Fusion protein in periplasm	0 to –20°C
pFLAG-CTC Expression Vector	Expression and secretion of Carboxy-Terminal FLAG Fusion protein in cytoplasm	
pFLAG-CTS-BAP Control Plasmid	Positive control for protein expression, detection and purification	
N-24 Primer	Sequencing of Amino-Terminus of Carboxy-terminal FLAG Fusion junction	0 to –20°C
C-24 Primer	Sequencing of Carboxy-Terminal FLAG Fusion junction	
ANTI-FLAG M2 Monoclonal Antibody	Immunological detection of Carboxy-terminal FLAG fusion protein from pFLAG-CTS or CTC Expression Vector	0 to –20°C
ANTI-FLAG M2 Affinity Gel	Affinity purification of Carboxy-terminal FLAG fusion protein from pFLAG-CTS or CTC Expression Vector	2 to 8°C
FLAG Peptide	Peptide elution of FLAG fusion protein from M2 Affinity Gel	2 to 8°C
C-Terminal FLAG-BAP Control Protein	Positive control for detection and purification	0 to –20°C

***For description see Footnotes to Table 4**

Table 4.

Components of the *E. coli* FLAG-Shift Expression Kit

Kit Component*	Function	Storage
pFLAG-Shift$_{12}$™ Expression Vector	Expression and secretion of Amino-Terminal FLAG-Shift Fusion protein in periplasm	0 to –20°C
pFLAG-Shift$_{12c}$™ Expression Vector	Expression of Met-Amino-Terminal FLAG-Shift Fusion protein in cytoplasm	0 to –20°C
pFLAG -Shift$_{12}$™ -BAP Control Plasmid	Positive control for protein expression, detection and purification	0 to –20°C
N-24 Primer	Sequencing of Amino-Terminal of FLAG-Shift Fusion junction	0 to –20°C
C-24 Primer	Sequencing of Carboxy-Terminus of FLAG-Shift Fusion junction	0 to –20°C
ANTI-FLAG M1 Monoclonal Antibody	Immunological detection of Amino-terminal FLAG fusion protein from pFLAG-Shift$_{12}$ Expression Vector	0 to –20°C
ANTI-FLAG M2 Monoclonal Antibody	Immunological detection of Met-Amino-terminal FLAG fusion protein from pFLAG-Shift$_{12c}$ Expression Vector and pFLAG Shift$_{12}$ Expression Vector	0 to –20°C
ANTI-FLAG M1 Affinity Gel	Affinity purification of Amino-terminal FLAG-Shift fusion protein from pFLAG-Shift$_{12}$ Expression Vector	2 to 8°C
ANTI-FLAG M2 Affinity Gel	Affinity purification of Amino-terminal FLAG-Shift fusion protein from pFLAG-Shift$_{12}$ Expression Vector Met-Amino-terminal FLAG-Shift fusion protein from pFLAG-Shift$_{12c}$ Expression Vector	
FLAG Peptide	Peptide elution of FLAG-Shift fusion protein from ANTI-FLAG M1 or M2 Affinity	2 to 8°C
N-Terminal FLAG-™ BAP Control Protein	Positive control for detection of Amino-terminal and Met-Amino-terminal Shift Fusion proteins	0 to –20°C
Enterokinase	Removal of FLAG peptide from Amino-terminal and Met-Amino-Terminal FLAG-Shift Fusion protein	0 to –20°C 0 to –20°C

* **The Expression Vectors** (10 µg) are supplied in 1 mM Tris-HCl, 0.1 mM EDTA (pH 8)
The Control Plasmids (1 µg) are supplied in 1 mM Tris-HCl, 0.1 mM EDTA (pH 8)
The N-26 and C-24 Primers (1 µg) are supplied in 10 mM Tris-HCl, 1 mM EDTA (pH 8)
The ANTI-FLAG Antibodies M1 and M2 (200 µg) are supplied in PBS with 0.02% sodium azide
The FLAG Peptide (4 mg) is supplied in a lyophilized form.
The ANTI-FLAG Affinity Gels (1 ml) are supplied in PBS with 0.02% sodium azide
The Control Proteins (100 µg) are supplied in a buffer containing 10 mM Tris-HCl, 120 mM NaCl, 0.05 mM ZnCl, 50% glycerol (pH 8)

COMPONENTS OF FLAG EXPRESSION SYSTEM

The components of the FLAG Expression System are summarized in **Table 1.** A brief description of the features of the Expression Vectors as well as suggestions for cloning DNA sequence of interest into these vectors is outlined below. Detailed maps of the vectors are included in the section "**Maps of Sigma *E. coli* Expression Vectors**" (page 49; **Figures 5-13**).

pFLAG-MAC and pFLAG-ATS Expression Vectors

Features of MCS

- **pFLAG-MAC (Figure 5) and pFLAG-ATS (Figure 6)** share common restriction sites between the vectors. Hence transfer of ORF between vectors is possible.
- Ten unique restriction sites represented in 3 reading frames. Pst I site between Kpn I and Bgl II site may be useful for mapping or excising an ORF. (Note: Xba I site is sensitive to methylation by dam methylase).
- Generate 5' overhangs, 3' overhangs or blunt ends upon digestion with a restriction enzyme.
- Translational stop codons in each frame but not within MCS.
- MCS compatible with C-terminal FLAG Expression vector.

Other Features

- pFLAG-MAC lacks the coding sequence for the OmpA signal peptide and is useful for the expression of Met-amino-terminal FLAG fusion proteins within the cytoplasm.
- pFLAG-ATS codes for the OmpA signal peptide that allows for expression of amino-terminal FLAG fusion proteins within the periplasm.

Cloning and Phasing an ORF into the MCS

The translational reading frame of an ORF to be sub-cloned into the *E. coli* FLAG Expression Vector is of primary importance in initial experimental design. **Table 5** serves as a helpful guide for comparison of the translational phases of each restriction site within the *E. coli* Amino-terminal FLAG Expression Vectors to the pUC18 cloning vector and Lambda gt11.

To minimize the background of clones lacking a desired insert, it is advisable to choose a cloning strategy that maximizes the efficiency of selecting insert-positive clones. This enrichment process requires additional work during the initial vector design and preparation but reduces the labor required for later screening steps, since a higher percentage of clones selected for evaluation will contain inserts. The enrichment procedure requires the scientist to perform a double-digest of the vector at the MCS. The two restriction sites must be selected to have at least one intervening restriction site. After digestion, annealing and ligation, the insert-containing vector will thus have lost one or more restriction sites originally present on the un-cut circular vector. A third restriction digest using a restriction enzyme specific for the deleted

sequences is then performed on the ligation mixture. Only the circular vector not containing insert DNA will be linearized by this additional restriction digest. The vector with insert will remain circular and will have a higher transformation efficiency than the linear vector, resulting in a higher representation of insert-positive clones.

Table 5.

Translational Phase of First Base of Each Restriction Site in the MCS of Amino-terminal FLAG Expression Vectors

Restriction Site	pFLAG-ATS pFLAG-MAC	pFLAG-1	pFLAG-2	pUC18	Lambda gt11
Tth III 1(GACN/NNGTC)	1		-		-
Hind III(A/AGCTT)	1		1	3	
Xho I (C/TCGAG)	1		1(Sal I also)		- (3 for Sal I)
Eco RI (G/AATTC)	3		1		3 (1 for Lambda)
Sma I (CCC/GGG)	2		-	1	
Xma I(C/CCGGG)		2		-	1
Kpn I (GGTAC/C)		3		-	2
Asp 718(G/GTACC)	3		1	2	
Bgl II (A/GATCT)		3		-	- (3 for BamHI)
Xba I (T/CTAGA)		3		-	3

Note: An ORF may be transferred between the pFLAG-MAC and pFLAG-ATS Expression Vectors since both share the same restriction sites in the same phase. An ORF may have to be re-phased if transferred from Lambda gt11, pUC18 or the pFLAG-1 and pFLAG-2 Expression Vectors. Phase 1 is in the same reading frame as the FLAG coding sequence.

Should the existing MCS not suffice for conveniently phasing a natural coding sequence, a Klenow fill routine, and subsequent vector recloning of any 5' protruding site changes the phase of all sites downstream to that position by +1. For example, a technique applicable to inserts bearing a 5' blunt end is to prepare the vector by (a) Klenow filling the Xma I site so that the 5' base of the blunt insert will be phase 1, or (b) Sma I cut the vector so that the 5' base of the blunt insert will be phase 2, or (c) Klenow filling either of the Tth III 1, Hind III or Xho I sites of the FLAG Expression Vector and re-ligate the blunt ended vector. The new vector is cut with Sma I so that the 5' base of a blunt insert will be phase three.

Klenow-fill protocol:

Cut the vector at a concentration of <200 µg/ml with the desired enzyme in the recommended buffer at 37°C in the presence of 0.03 U/µl Klenow and 50 µM each dNTP for >30 min. Heat inactivate the enzymes at 70-75°C for 30 min.

The Tth III 1 site in the MCS of the pFLAG-ATS and pFLAG-MAC Expression Vectors confers a special design feature that allows for manipulation of the first amino acid following the FLAG peptide. Direct cloning of an insert at Tth III 1 dictates Val as the first amino acid. However, a Klenow-fill blunting of the Tth III 1 site gives more freedom of first amino acid choice. The inserted gene sequence must start with an A or G to complete the final Lys codon of the FLAG coding sequence. This operation is compatible with many blunt enzymes whose 5' end is A or

G. A Tth III 1 cut, Klenow fill and recircularization will re-construct the vector as Tth III 1 minus, and phase all subsequent sites by +1, without changing the DNA sequence of the FLAG codon. Direct cloning into the Hind III site dictates Val-Lys-Leu as the first three amino acids.

pFLAG-CTS and pFLAG-CTC Expression Vectors

Features of the MCS

- **The pFLAG-CTC (Figure 7) and pFLAG-CTS (Figure 8)** Expression Vectors have a MCS similar to the pFLAG-ATS and pFLAG-MAC Expression Vectors.
- The MCS contains restriction sites represented in all three reading frames.
- Restriction digestion generate 5' overhang, 3' overhang or blunt ends following digestion with the appropriate restriction enzyme.

Other Features

- There is a translational stop codon immediately downstream, but not within, the MCS. This stop codon is in phase with the coding sequence of the adjacent FLAG peptide.

Cloning and Phasing an ORF in the MCS

It is important to note that any ORF cloned into a Carboxy-terminal FLAG Expression Vector must be phased with respect to: **(1)** The ATG translational start codon immediately upstream of the MCS and **(2)** The C-terminal FLAG DNA coding sequence immediately downstream of the MCS. Any insert must be free of stop codons to insure proper read-through to the C-terminal FLAG peptide.

Table 6.

Translational Phase of First Base of Each Restriction Site in the MCS of the pFLAG-CTC and pFLAG-CTS Expression Vectors

Restriction Site	ATG Start Codon		FLAG Peptide	
	pFLAG-CTC	pFLAG-CTS	pFLAG-CTC	pFLAG-CTS
Nde I (CA/TATG)	1	-	2	-
Hind III (A/AGCTT)	1	2	2	2
Xho I (C/TCGAG)	1	2	2	2
Eco RI (G/AATTC)	3	1	1	1
Sma I (CCC/GGG)	2	3	3	3
Xma I (C/CCGGG)	2	3	3	3
Kpn I (GGTAC/C)	3	1	1	1
Asp 718 (G/GTACC)	3	1	1	1
Bgl II (A/GATCT)	3	1	1	1
Sal I (CG/TCGAC)	3	1	1	1

pFLAG-1™ and pFLAG-2™ Expression Vectors

(These vectors are not supplied in the Kit, but can be purchased separately)

Features of MCS

- **pFLAG-1 (Figure 9)** and **pFLAG-2 (Figure 10)** have identical MCS that allow transfer of ORF between the vectors
- MCS contain 6 unique restriction sites
- Restriction enzyme digestion generate 5', overhangs
- Translational stop codon immediately downstream of the MCS in the same reading frame as the coding sequence of the FLAG peptide and two out of phase with the FLAG peptide.

Other Features

- pFLAG-1 encodes the gene sequence for the OmpA signal peptide for expression of amino-terminal FLAG fusion proteins within the periplasm.
- pFLAG-2 lacks the coding sequence for the OmpA signal peptide allowing the expression of a Met-amino-terminal FLAG fusion protein within the cytoplasm.

pFLAG-Shift$_{12}$ and pFLAG-Shift$_{12c}$ Expression Vectors

Features of the MCS

- The pFLAG-Shift$_{12}$ **(Figure 11)** and pFLAG-Shift$_{12c}$ (Figure 12) Expression Vectors share identical multiple cloning sites and differ only with respect to the presence (pFLAG-Shift$_{12}$) or absence (pFLAG-Shift$_{12c}$) of the DNA coding sequence for the OmpA signal peptide.
- The restriction sites are compatible with those of pFLAG-MAC and pFLAG-ATS Expression Vectors.
- Restriction sites are represented in all reading frames and they generate 5' overhang, 3' overhang and blunt ends on restriction enzyme digestion.
- There are translational stop codons in each of 3 reading frames immediately downstream, but not within, the MCS.

Note: The Xba I site is sensitive to dam methylation. Use a dam minus host strain if this site is required.

Applications of FLAG-Shift Vectors

The FLAG-Shift Expression Vectors[17] are useful for the expression of an ORF whose phase is not known. This is generally the case for clones within a shotgun, cDNA or genomic library. Expression of the ORF by a FLAG-Shift Expression Vector permits translational frameshifting[31-35] at the junction of the FLAG sequence and a cloned ORF. Frameshifting is due to a translational "shift" sequence of twelve thymidines situated between the FLAG coding sequence and the cloned ORF. Sufficient frameshifting occurs to cause productive expression of the cloned ORF as a part of the FLAG fusion protein. This ORF is represented in the proper phase of the FLAG fusion protein regardless of the reading frame in which it was initially cloned. An ORF can be interchanged between the vectors since they share identical restriction sites in the same phase.

Other Characteristics of the Vector Backbone

The *tac* Promoter:

A DNA coding sequence is expressed in an *E. coli* host by virtue of the tac promoter[25]. This promoter is composed of a hybrid between the –35 consensus sequence of the *trp* promoter and the –10 consensus sequence of the *lac* promoter. The *tac* promoter is among the most productive and proficient promoters used for overproducing fusion proteins in *E. coli.* The *lac* operator is the binding site of the *lacI* repressor protein. The *lacI* repressor protein maintains transcriptional repression of the FLAG fusion protein. The *lacI* repressor is encoded on the same plasmid, and is constitutively produced by the *lacIq* promoter (a mutated, more productive promoter for *lacI)*. The FLAG fusion protein is produced by induction. Induction that occurs by addition of isopropylthio-ß-galactoside (IPTG) to the growing cell transformed with a FLAG Expression Vector encoding the *lac* operator. IPTG specifically binds the *lacI* repressor protein and prevents the repressor from binding to the operator.

It is important that potentially lethal gene products under the control of a productive promoter be maintained in a repressed mode. In addition, overproduction of the gene product might burden the host cell capacity to maintain normal growth and maintenance functions in the absence of repression. The *tac* promoter is clearly sufficient for this purpose, since many genes have been successfully cloned and overexpressed using this promoter. However, the *tac* promoter may not always be completely repressed[26] and, therefore, may not always be applicable to the cloning of some lethal gene products.

Translational Initiation Site:

This includes the consensus sequence for the Shine-Dalgarno Ribosome binding site. This is optimally spaced with regard to the ATG translational start codon.

OmpA Signal Peptide:

The 63 base pair, DNA coding sequence for the 21 amino acid OmpA signal peptide[27] is contained in the pFLAG-ATS, pFLAG-Shift$_{12}$, pFLAG-CTS and pFLAG-1 Expression Vectors. **(Figures 6,8,9,11).** The OmpA signal sequence is normally responsible for the secretion of OmpA (Outer membrane protein A), an abundant protein in *E coli.* The OmpA signal peptide is cleaved from the FLAG fusion protein by a signal peptidase as it crosses the inner, cytoplasmic membrane into the periplasmic space.

Translational Stop Codons:

Translational stop codons in all three reading frames are located downstream of the MCS in all amino-terminal FLAG and FLAG-Shift Expression Vectors. Only one stop codon is present in carboxy-terminal FLAG expression vectors. This stop codon is located immediately downstream, and in phase with the FLAG DNA coding sequence. There are no stop codons within the MCS of any *E. coli* FLAG Expression Vector except the pFLAG-1 and pFLAG-2 Expression Vectors. The MCS of these expression vectors contains two stop codons that are out of phase with the coding sequence for the FLAG peptide.

Transcriptional Terminators:

Transcription of a cloned gene is actively terminated downstream of the MCS by the *E. coli* ribosomal RNA transcription terminator (rrnB)[28]. There are two, tandem terminators, T1 and T2, in the termination signal.

Selectable Marker:

The *E. coli* FLAG Expression Vectors have a selectable marker amp^r (for ampicillin resistance) which allows for identification of a successfully transfected *E. coli* host. Resistance to ampicillin (amp^r) is encoded by the ß-lactamase gene in the plasmid. A culture of *E. coli* bearing a FLAG Expression Vector may be selected for by adding 50-100 µg/ml of ampicillin to growth medium.

Replication Origins:

pORI
The FLAG Expression Vectors are circular, episomal elements that are maintained in an *E. coli* host by the *colEI* origin of double stranded replication. Double stranded DNA is useful for cloning, expression, restriction mapping and sequencing.

f1-ori

An f1 origin of replication[29] is encoded in the plasmid for production of single-stranded DNA. The FLAG vectors were designed with this feature to aid in cloning or single stranded DNA sequencing. Circular, single-stranded FLAG DNA may be produced by superinfection of an appropriate *E. coli* host with the M13KO7[30] Helper Phage. The M13KO7 Helper Phage diverts control of replication to f1-ori. Selection for the helper phage is with kanamycin. The top or "+" strand of the FLAG Expression Vectors is that which is produced in the single-stranded form. (The positive (+) strand contains the 24 base pair DNA coding sequence for the FLAG marker peptide.)

FLAG-BAP Positive Control Plasmids

The FLAG Positive Control Plasmids express the BAP (Bacterial Alkaline Phosphatase) gene as a 49 kD FLAG fusion protein. The plasmids are useful as positive controls for the expression, affinity purification and detection of other FLAG fusion proteins. **(Figure 13.)**

pFLAG-ATS-BAP **(Figure13)** is a control for an ORF expressed in the pFLAG-ATS or pFLAG-MAC Expression Vectors; pFLAG-1-BAP **(Figure 13a)** is a control, available separately only, for an ORF expressed in the pFLAG-1 or pFLAG-2 Expression Vectors (neither expression vector is offered in kits, but are available separately); pFLAG-CTS-BAP **(Figure 13b)** is the appropriate control for an ORF expressed in the pFLAG-CTS or pFLAG-CTC Expression Vectors. pFLAG-Shift-BAP **(Figure 13c)** serves as a control for an ORF expressed by either the pFLAG- $Shift_{12}$ or pFLAG-$Shift_{12c}$ Expression Vectors. Each positive control plasmid contains the same backbone as the *E. coli* FLAG Expression Vectors (**Figures 5 to 12**).

N-26 and C-24 Oligodeoxyribonucleotide Primers

The N-26 and C-24 oligodeoxyribonucleotide primers are provided for sequencing either the amino- or carboxy-terminal FLAG fusion junctions of the inserted gene sequence. The N-26 and C-24 primers are applicable for use in cycle sequencing. They may also be useful in amplification techniques for gene modification of FLAG fusion proteins.

The N–26 primer is a twenty six base oligodeoxyribonucleotide which binds to the negative DNA strand at map position 1 - 26. This binding site is immediately upstream of the *tac* promoter of all *E. coli* FLAG Expression Vectors **(Figures 5 to 13).** The N-26 primer is useful for sequencing amino-terminal FLAG fusion junctions encoded by inserts cloned into the pFLAG-MAC, pFLAG-ATS, pFLAG-1, pFLAG-2, pFLAG-$Shift_{12}$ and pFLAG-$Shift_{12c}$ Amino-terminal Expression Vectors **(Figures 5, 9 to 12).**

The C-24 primer is a twenty four base oligodeoxyribonucleotide which binds to the positive, FLAG coding DNA strand. The binding site is located immediately downstream of the MCS between the translational stop codon(s) and the transcriptional terminators of all FLAG Expression Vectors **(Figures 5 to 13).** The C-24 primer is useful for sequencing carboxy-terminal FLAG fusion junctions encoded by inserts cloned into the pFLAG-CTS and pFLAG-CTC Carboxy-terminal Expression Vectors **(Figures 7, 8).**

Other components required for the purification and detection of the fusion proteins are discussed in the subsequent sections.

CONFIRMATION OF FUSION JUNCTION OF CLONED ORF

The DNA sequence of candidate FLAG clones must be verified at the FLAG fusion junction for proper phasing. While restriction analysis is necessary, it is usually not sufficient to confirm the detailed sequence of the FLAG fusion junction. This involves the following steps:

- **Isolation of Single Strand DNA**
- **Sequencing of DNA**

Isolation of Single Strand FLAG DNA

Growth Media

M9 MEDIUM
COMPOSITION FOR 1 LITER (sterile solution)

200 ml of ***5x M9 salts**
20 ml of 20% glucose (sterile)
750 ml of deionized water (sterile)

*** 5x M9 salts (1 liter, sterile solution)**
64 g Sodium phosphate (Di-sodium salt)
15 g Potassium phosphate (Mono potassium salt)
25 g NaCl
5 g Ammonium Chloride

2x YT MEDIUM
FOR 1 LITER STERILE SOLUTION

16 g Bacto Tryptone
10 g Yeast Extract
5 g NaCl

Single-strand FLAG DNA[13] is useful for manipulation of cloned genes in *E. coli* FLAG Expression Vectors. The FLAG expression vectors are phagemids containing the phage f1 origin of replication[29]. The f1-ori allows production of extracellular phage particles containing single-stranded DNA. Phagemid particles contain the positive, coding strand of the FLAG Expression Vector (the DNA strand that contains the coding sequence for the FLAG peptide). Phagemid particles are generated by superinfection of *E. coli* hosts carrying a FLAG Expression Vector with the M13K07[30] helper phage. The following protocol describes the generation and purification of single stranded DNA from FLAG phage particles. Single stranded DNA is useful for DNA manipulation or sequencing. Only the C-24 primer will bind in the proper orientation for

sequencing the C-terminus of ORFs cloned into the MCS of FLAG Expression Vectors. It is essential that the *E coli* host possess an F' genotype for production of single-stranded DNA.

1. Plate *E. coli* cells carrying your ORF in a FLAG Expression Vector on M9 medium + 50 µg/ml Ampicillin. This medium selects for the F' phenotype necessary for single strand production.

2. Grow cells overnight at 37°C.

3. Add 2 ml of 2xYT containing 250 µg/ml Ampicillin to the surface of the plate. Suspend bacteria.

4. Add 1 ml of the cell suspension to 10 ml of 2xYT containing 250 µg/ml Ampicillin. Grow until OD_{600} is between 0.4 - 0.6.

5. Dilute the culture 1/50 in 50 ml 2xYT + 250 µg/ml Ampicillin in a 500 ml flask. Shake at 37°C for 30 min. Take an OD_{600} to estimate the number of cells/ml.

6. Add M13K07 Helper phage to a Multiplicity of Infection (MOI) of 20. After 30 min growth, add 70 µl of 50 mg/ml Kanamycin. Continue shaking for 6 hours. Longer growth periods may result in deletions within the DNA.

7. Pellet cells at 17,000 x *g* for 30 min. Recover supernatant and filter. For each ml of supernatant add 0.25 ml of 20% PEG-6000 in 3.5 M Ammonium acetate. Mix well by inversion and place on ice for 2-48 hours.

8. Centrifuge as in step **7.** Decant supernatant. A small white pellet should be visible.

9. Resuspend in 300 - 700 µl of TE by vortexing. Transfer to a microfuge tube.

10. Add an equal volume of freshly distilled phenol. Vortex 1-2 min. Spin and recover the aqueous phase without disturbing the white interface.

11. Extract with an equal volume of 1:1 phenol/chloroform (Chloroform is 24:1 $CHCl_3$: isoamyl alcohol). Continue extractions 4 - 6 times until a slight white interface is seen.

12. Extract with an equal volume of 24:1 $CHCl_3$: isoamyl alcohol. Remove upper aqueous phase.

13. Precipitate the aqueous phase by adding 0.5 volumes of 7.5 M Ammonium Acetate and 2 volumes of ethanol. Precipitate overnight at -20°C or 30 min at -70°C. Wash pellet with 70% ethanol. Air dry or dry down in a speed vac.

14. Resuspend DNA in 20 - 40 µl distilled, deionized water.

Confirmation of FLAG Fusion Junctions

Buffer:

Following identification of a clone with the desirable insert, it is preferable to isolate the DNA and confirm the sequence of the FLAG fusion junction before proceeding further.

The N-26 primer is used for sequencing DNA at the 5' end of the ORF. This corresponds to the amino-terminal fusion junction of FLAG fusion proteins. It can also be used to sequence the amino-terminus of carboxy-terminal FLAG fusion proteins. The N-26 sequencing primer binds immediately 5' to the *tac* promoter at position 1-26 of the negative DNA strand. The negative strand is the strand complementary to the strand containing the FLAG DNA coding sequence in all FLAG Expression Vectors. It has the following sequence:

N-26 Primer: 5' - CAT CAT AAC GGT TCT GGC AAA TAT TC - 3'

Sequencing of N-terminal FLAG fusion junctions

The DNA sequence of the *tac* promoter and ribosome binding site in all FLAG Expression Vectors contains secondary structure. Secondary structure may lead to band compressions and other artifacts on the DNA sequencing gel. For this reason, sequencing both strands using 7-deaza-dGTP is recommended when confirming amino-terminal FLAG fusion junctions or sequencing the amino-terminus of carboxy-terminal FLAG fusion proteins.

The C-24 primer is used for sequencing the 3' end of the ORF corresponding to the carboxy-terminal FLAG fusion junction of FLAG fusion proteins expressed by *E. coli* FLAG Expression Vectors. However, it can be used to sequence the carboxy-terminus of amino-terminal FLAG fusion proteins. The C-24 sequencing primer binds immediately downstream of the MCS to the positive DNA strand (the strand that contains the FLAG DNA coding sequence) in all FLAG Expression Vectors. The C-24 primer has the following sequence:

C-24 Primer: 5' - CTG TAT CAG GCT GAA AAT CTT CTC - 3'

Sequencing of C-Terminal FLAG fusion junctions

Table 7 lists the distances of the 3' end of the N-26 primer from the 5' end of the MCS and the distance of the 3' end of the C-24 primer from the 3' end of the MCS in each of the *E. coli* FLAG Expression Vectors. Also, see maps of ***E. coli* FLAG Expression Vectors** in the back of this manual.

Table 7.

Distances in Base Pairs of FLAG Primers from the 5' & 3' ends of of E. Coli FLAG Expression Vectors

	N-26 Primer (5' End)	C-24 Primer (3' End)
pFLAG-ATS	**172**	**21**
pFLAG-MAC	**112**	**21**
pFLAG-CTS	**202**	**48**
pFLAG-CTC	**150**	**48**
pFLAG-Shift-$_{12}$	**186**	**21**
pFLAG-Shift-$_{12c}$	**126**	**21**

Double Strand Sequencing with the N-26 and C-24 Primers

The following protocol is designed to prepare sufficient primer:template for 2 sets of 4 sequencing reactions: 2G, 2A, 2T and 2C reactions; using either the N-26 or C-24 primers in sequencing. The first section describes preparation of denatured template by alkaline denaturation with NaOH. The second section describes the protocol for hybridization of either the N-26 or C-24 primer to a FLAG template. The primers are intended for sequencing short stretches of DNA at FLAG fusion junctions. However, they have been used to generate 400 bases of useful DNA sequence using standard methods.

Preparation of Denatured FLAG DNA Template:

1. Denature 7 µg of highly purified FLAG DNA in 70 µl of 1xTE (10 mM Tris-HCl, 1 mM EDTA at a final pH of 8.0) by adding 3 µl (1/25 volume) of 5M NaOH at 37°C for 5 min.

2. Precipitate denatured FLAG DNA for 30 min at room temperature by adding 150 µl (2 volumes) of premixed **potassium acetate/isopropanol** made as follows:

Recipe for 400 ml Potassium Acetate/Isopropanol:
For 100 ml Potassium Acetate add:
60 ml of 5 M potassium acetate
11.5 ml glacial acetic acid
28.5 ml of deionized water to final volume of 100 ml

Add 300 ml isopropanol to the pre-mixed potassium acetate solution.

3. Collect precipitated and denatured FLAG DNA by centrifugation at 10,000 x *g* for 5 min.

4. Wash the FLAG DNA pellet with 1 ml ethanol and dry.

5. Resuspend FLAG DNA in 20 µl of 1xTE. The final concentration should be 0.35 µg/µl. You can store the irreversibly denatured FLAG DNA template at -20°C at this point, if desired.

Priming FLAG DNA Template with N-26 or C-24 Sequencing Primer:

The following protocol uses 10 µl or half of the preceding preparation of irreversibly denatured FLAG DNA template. This is sufficient for 1 set of 4 sequencing reactions (1G, 1A, 1T and 1C reaction).

1. Dilute the sequencing primer from an initial concentration of 5 pmol/µl to a final concentration of 1.67 pmol/µl by removing a 3 µl aliquot of N-26 sequencing primer from the vial and adding 6 µl of 1xTE to a final volume of 9 µl.

2. Add 2-3 µl (3.5-5 pmoles) of Sequencing Primer to 10 µl (1 pmol) of denatured FLAG DNA template.

3. Heat the 12-13 µl of primer:FLAG DNA template mixture in an appropriate volume of your sequencing buffer to make the buffer 1X at 70°C in a wet temperature block for 2 min.

4. Slow cool the mixture by placing the temperature block at room temperature for about 20 min.

5. Distribute the primed FLAG DNA template to 4 tubes to be used in the G, A, T and C DNA sequencing reactions. The primer:FLAG template is now ready for sequencing.

Hosts for Protein Expression

All *E. coli* FLAG Expression Vectors contain the coding sequence for the *lacI* repressor protein. All *E. coli* FLAG Expression Vectors allow complete freedom of choice of a specific host cell strain for protein expression using the *tac* promoter. However, it is best to select a host that is also *recA*. This will minimize rearrangements of your DNA because of recombination events. Recombination events may cause problems in protein expression later on. Successful protein expression will depend primarily on the properties of the protein itself.

An empirical selection of protein / host combination is recommended if no experience using a specific host strain has been gained. Protease negative strains can minimize or eliminate proteolytic degradation of a fusion protein. However, no one strain is guaranteed to solve a proteolysis problem.

The most commonly used host strains successfully employed as a starting point for FLAG protein expression have been: DH5α and BL21 (a protease negative strain) which are readily available hosts through the ATCC in Maryland. We successfully express our model FLAG fusion protein, FLAG-BAP, in host strains LE392, LL308 or JM103. The LE392 & JM103 strains are available through the same source. However, this is not to be construed as our recommendation as a host for every FLAG fusion protein.

PROTOCOL FOR EXPRESSION OF FLAG FUSION PROTEIN

Screening Transformants for FLAG Fusion Proteins

- Use Anti-M2 Monoclonal antibody to screen the presence of fusion as this antibody will bind to the FLAG marker at any location on FLAG fusion protein.

- Confirm the presence of the expressed protein with expected molecular weight by SDS-PAGE or Western blot.

Use the following controls:

Negative control: Proteins from a FLAG Expression Vector without a cloned ORF
Positive control: FLAG BAP positive control protein for monitoring the integrity of the M2 Mab as well as the other components of the detection system.

Protocol for Growing *E. coli:*

Growth Medium:
***LB MEDIUM* FOR 1 LITER STERILE SOLUTION**
10 g Bacto Tryptone
10 g NaCl
5 g Bacto Yeast Extract

Buffer:
SDS PAGE SAMPLE BUFFER
400 mM DTT
200 mM Tris-HCl (pH 6.8)
40% glycerol
8% SDS
0.4% Bromophenol Blue

1. Inoculate individual colonies into 5 ml LB containing 50 µg/ml Ampicillin and 0.4% glucose. Incubate overnight at 37°C

2. Dilute overnight cultures 1:100 into 5 ml prewarmed LB containing 50 µg/ml Ampicillin and 0.4% glucose in a 125 ml flask to insure good aeration. Incubate with shaking at 37°C.

3. At OD_{600} of 0.2 remove 0.5 ml of pre-induction culture to a microfuge tube. Pellet cells and resuspend pellet in 25 µl of SDS-PAGE sample buffer and heat to 95°C for 5 min. To the remaining culture add IPTG to 0.5 mM. Continue growth at 37°C for 2 hours.

4. At 2 hours post induction, remove a 0.5 ml aliquot, pellet in a microfuge, resuspend pellet in 100 µl of SDS-PAGE sample buffer and heat to 95°C for 5 min.

5. Analyze pre- and post-induction samples by SDS-PAGE or by Western blot (see page 39) using the ANTI-FLAG M2 Monoclonal Antibody.

Optimization of Expression of Fusion Protein

A number of factors determine the optimal expression of the fusion protein. Some of the key conditions that influence the expression are:

- Host strain used for expression
- Biochemical properties of the FLAG fusion protein and its physiological impact on the host strain
- Time of initial induction
- Temperature of induction
- Length of induction.

We find that the optimal conditions for expression of the FLAG-BAP fusion protein in the LE392 host includes growth at 37°C into log phase (OD_{600} = 1.0) in LB broth containing 0.4% glucose and 50 µg/ml Ampicillin. Growth to log phase is followed by induction with 500 µM IPTG for 2 hours. These conditions should not be interpreted as our recommended conditions for other FLAG fusion proteins.

1. Inoculate an overnight culture of *E. coli* expressing the cloned FLAG fusion protein in LB containing 50 µg/ml Ampicillin and 0.4% glucose at 37°C.

2. Dilute the overnight culture 1:100 into 20 ml of LB containing 50 µg/ml Ampicillin and 0.4% glucose. Continue growth at 37°C until an OD_{600} of 0.2-1.0. You may want to vary the OD and temperature for induction since this may improve expression levels.

3. Remove a 0.5 ml aliquot of pre-induction culture and pellet in a microfuge. Resuspend the pellet in 25 µl of SDS-PAGE sample buffer. Heat to 95°C for 5 min. and store frozen.

4. To the remaining culture, add IPTG to a final concentration of 500 µM and continue growth at 37°C.

5. At 2, 4, and 6 hours post-induction, determine the OD_{600} and remove an aliquot of culture. Dilute in LB to an OD equal to the OD at initial induction with IPTG. Remove 0.5 ml to a microfuge tube and pellet cells in a microfuge. Add 25 µl of SDS-PAGE sample buffer to the pellet. Heat to 95°C for 5 min and store frozen.

6. Determine protein expression levels by analysis of pre- and post-induction samples by Western blot using the ANTI-FLAG M2 Monoclonal Antibody.

7. Vary the induction conditions or host if you wish to improve expression levels further.

Note: *If protein expression is not satisfactory on a first attempt, it is preferable to optimize the induction conditions before transforming into a new host.*

ISOLATION OF FLAG FUSION PROTEINS

Analytical Fractionation of FLAG Fusion Proteins

The following protocol will determine whether the FLAG fusion protein fractionates to the periplasm **(Step 9),** soluble whole cell fraction **(Step 15),** insoluble, whole cell fraction **(Step 16)** or to the culture medium **(Step 4c)** of *E. coli*.

The results from fractionation will indicate whether to perform preparative isolation procedures using the: **(1)** Osmotic shock procedure, **(2)** whole cell extraction, **(3)** whole cell extraction followed by solubilization of the FLAG fusion protein with detergents or chaotropes or **(4)** buffer exchange of culture supernatants by gel filtration.

All steps in the protocol should be followed if an ORF was cloned into the pFLAG-1, pFLAG-ATS, pFLAG-CTS or pFLAG-Shift$_{12}$ Expression Vectors for periplasmic secretion. If the amino-terminal FLAG fusion protein expressed by the pFLAG-1, pFLAG-ATS or pFLAG-Shift$_{12}$ Expression Vector fractionates to the periplasm, then proceed to preparative isolation by Osmotic Shock in the next section. Following osmotic shock, protein purification is performed using EDTA chelation with the ANTI-FLAG M1 Affinity Gel or competitive elution with FLAG peptide using the ANTI-FLAG M2 Affinity Gel. Purification of FLAG fusion proteins expressed and secreted to the periplasm by the pFLAG-CTS Expression Vector can only be purified with the ANTI-FLAG M2 Affinity Gel. **(Figure 3 and Table 1.)**

If an ORF was cloned into the pFLAG-2, pFLAG-MAC, pFLAG-CTC or pFLAG-Shift$_{12c}$ Expression Vectors then only cytoplasmic expression is likely. In this case, the osmotic shock procedure is not necessary and only steps **1 - 4** and **10 - 15** in the protocol should be followed. If the protein fractionates to the soluble whole cell fraction, then proceed to preparative isolation via whole cell extraction. This is followed by peptide elution with the ANTI-FLAG M2 Affinity Gel. **(Figure 3 and Table 1).** If the protein localizes to the insoluble fraction, then it is necessary to solubilize with a detergent or chaotrope prior to affinity purification with the ANTI-FLAG M2 Affinity Gel. If solubilization is required, it is important to use an agent that is compatible with the ANTI-FLAG M1 or M2 Affinity Gel.

There are a number of situations in which foreign proteins expressed in *E. coli* will not be secreted into the periplasm. For example, when expressed in *E. coli*, many eukaryotic proteins form insoluble inclusion bodies[36-38]. Inclusion bodies may be caused by misfolding due to improper disulfide crosslinking or glycosylation. Glycosylation and disulfide crosslinking may be required for stability and solubility of the protein. The FLAG marker is a hydrophilic, low molecular weight peptide that is unlikely to cause misfolding. In addition, if your protein is a membrane or receptor protein then it is likely that it contains hydrophobic domains and may not traverse the *E. coli* inner plasma membrane when co-expressed with OmpA.

If your protein does localize to the insoluble whole cell fraction, it may be necessary to induce at a different temperature[38] or to solubilize with a detergent or chaotropic agent[36-37].

Whole Cell Sample

1. Inoculate an overnight culture at 37°C or previously determined optimal temperature in LB containing 50 µg/ml Ampicillin and 0.4% glucose. Note: For some proteins, secretion may be more efficient at 30°C. Also, some insoluble proteins may be soluble at 23°C.

2. Dilute 1:100 into 100 ml of LB containing 50 µg per ml Ampicillin and 0.4% glucose in a 500 ml flask. Shake at 37°C until OD_{600} = 0.2 or the previously determined optimum induction OD.

3. Add IPTG to 500 µM and continue growth at 37°C for 2 hours or the previously determined induction time. Remove a 0.5 ml aliquot, microfuge and decant supernatant. Resuspend pellet in 25 µl SDS-PAGE sample buffer, heat to 95°C for 5 min and store the whole cell sample frozen.

4. Divide the remaining culture into 2 aliquots and centrifuge each aliquot at 5,000 x *g* for 10 min. at 10°C to pellet the cells.

(a) This pellet will be used to determine fractionation of the FLAG fusion protein to the periplasmic space by the osmotic shock procedure **(Steps 5-9).** Note: This sample must not be frozen, since freeze-thaw cycles are known to cause whole-cell lysis. Periplasmic proteins will thus be contaminated with cytoplasmic proteins.

(b) The second pellet will be used for fractionation of the whole cell extract **(Steps 10-15)** into soluble **(Step 14)** or insoluble **(Step 15)** fractions. Freeze this pellet at -70°C until ready for whole cell fractionation.

(c) Save 1 ml of the culture supernatant from **(a)** or **(b)** at -70°C. This sample will be analyzed by Western blot to determine if the FLAG fusion protein fractionates to the culture media.

Periplasmic Fraction by Osmotic Shock

Buffer for Osmotic Shock (USE 40 ml/g of cells)
500 mM Sucrose
30 mM Tris-HCl (pH 8)
1 mM EDTA

5. Warm the pellet in **(4a)** to room temperature and re-suspend in 40 ml/g cells of 0.5 M Sucrose, 0.03 M Tris, 1 mM EDTA at a final pH of 8.0 per gram of cells. Omit this sample for an ORF expressed without the OmpA signal peptide from the pFLAG-2, pFLAG-MAC, pFLAG-CTC or pFLAG-$Shift_{12c}$ Expression Vectors and proceed **to Step 10.**

6. Centrifuge at 3,500 x *g* for 10 min at 10°C for osmotic shock.

7. Decant the supernatant and rapidly resuspend in 25 ml/g cell pellet ice-cold, distilled water.

8. Centrifuge at 3,500 x *g* for 10 min at 4°C.

9. Collect supernatant immediately. Mix 50 µl supernatant with 50 µl SDS-PAGE sample buffer, heat to 95°C for 5 min, and freeze the periplasmic fraction until ready for electrophoresis. Freeze the remaining supernatant, if desired.

Whole Cell Extract: Soluble and Insoluble Fractions

Buffers:

Extraction Buffer A

50 mM Tris-HCl, pH 8.0
5 mM EDTA
0.25 mg/ml lysozyme
50 µg/ml sodium azide

Extraction Buffer B

1.5 M NaCl
0.1 M $CaCl_2$
0.1 M $MgCl_2$
0.02 mg/ml DNase I
0.05 mg/ml ovomucoid protease inhibitor

10. Thaw the whole cell sample **(4b)** at room temperature.

11. Add 5 ml of ***Extraction Buffer*** *A.* Incubate at room temperature for 5 min or until cells are lysed (sample should become viscous). Note: Use of lysozyme to lyse *E. coli* cells will result in a strong protein band in an SDS gel at about 14 kD. If this band interferes with subsequent analyses, sonication to disrupt the cells is recommended.

12. Add 0.5 ml of ***Extraction Buffer B***. Incubate at room temperature for 5 min or until no longer viscous.

13. Centrifuge at 18,000 x *g* for 0.5 hour.

14. Collect supernatant containing the soluble, whole cell fraction. Mix 50 µl of supernatant with 50 µl of SDS-PAGE sample buffer, heat to 95°C and store frozen. The remaining suspension may be stored frozen, if desired.

15. Resuspend the pellet containing insoluble, whole cell material in 5ml of ***Extraction Buffer A***. Mix 50 µl with 50 µl SDS-PAGE sample buffer, heat to 95°C for 5 min and store frozen. The remaining suspension may be stored frozen, if desired.

Analyze the whole cell fraction **(Step 3);** culture medium sample **(Step 4c);** periplasmic sample **(Step 9);** whole cell soluble fraction **(Step 14)** and whole cell insoluble fraction **(Step 15)** by

SDS-PAGE or Western blot next to the appropriate controls using the ANTI-FLAG M2 Monoclonal Antibody.

If the FLAG fusion protein is found primarily in the periplasm **(Sample 9),** preparative isolation may be carried out by the osmotic shock procedure described in the next section prior to affinity purification. If the FLAG fusion protein is found in the soluble whole cell fraction **(Sample 14),** but not the periplasmic sample, preparative isolation should be carried out from whole cell extracts described below. If the FLAG fusion protein is localized to the whole cell insoluble fraction **(Sample 15),** solubilize the FLAG fusion protein from the preparative, whole cell extract with a detergent prior to purification with the ANTI-FLAG M1 or M2 Affinity Gel. (Please see section on detergents in section on immuno-affinity purification of FLAG Fusion Proteins). If your protein fractionated into the cell medium **(Sample 4c),** the supernatant should be desalted on a Sephadex G-25 column into the appropriate column loading buffer (TBS or TBS/Ca). Add protein cofactors and/or protease inhibitors to the exchange buffer if necessary.

PREPARATIVE ISOLATION OF FLAG FUSION PROTEINS

Osmotic Shock Procedure for Isolation of Periplasmic Proteins

The following osmotic shock procedure is recommended for preparative isolation of FLAG fusion proteins that are secreted to the periplasmic space.

1. Grow cells according to optimized growth and induction conditions.

Note: *See previous section on "Optimization of Expression" for optimal conditions for FLAG fusion protein growth and expression.*

2. Harvest cells by centrifugation at 5,000 x *g* for 10 min. at 10°C.

3. Resuspend cells in 10 mM Tris-HCl (pH 8.0) diluted from a 0.1 mM stock with room temperature distilled water. Use 40-80 ml/gram cells.

4. Centrifuge mixture at 3,500 x *g* for 10 min at 10°C.

5. Decant supernatant and resuspend pellet in 10 mM Tris-HCl (pH 8.0) at room temperature.

6. Centrifuge at 3,500 x *g* for 10 min. at 10°C.

7. Resuspend cell pellet in 0.5 M Sucrose, 0.03 M Tris (pH 8.0), 1 mM Na_2EDTA at room temperature. Use 40 ml/gram of cells.

8. Centrifuge at 3,500 x *g* for 10 min. at 10°C.

9. Decant supernatant and rapidly resuspend cell pellet in 25-35 ml ice-cold distilled water/gram of cell pellet.

10. Centrifuge mixture at 3,500 x *g* for 10 min at 4°C.

11. Collect supernatant immediately.

12. Add an equal volume of 2x concentrated TBS (pH 7.4) to the supernatant.

13. Add $CaCl_2$ to a final concentration of 1 mM. $CaCl_2$ may be omitted during purification with the ANTI-FLAG M2 Affinity Gel.

14. Centrifuge mixture at 25,000 x *g* for 60 min at 4°C.

15. Collect supernatant immediately.

16. Filter supernatant through Whatman No 1 filter paper.

17. Apply supernatant to the ANTI-FLAG M1 or M2 Affinity Gel.

Whole Cell Extracts of FLAG Fusion Proteins

Buffers:
Extraction Buffer A:

50 mM Tris (pH 8)
5 mM EDTA
0.25 mg/ml lysozyme
50 µg/ml Sodium Azide

Extraction Buffer B:

1.5 M NaCl
100 mM Calcium Chloride
100 mM Magnesium Chloride
0.02 mg/ml DNase1
50 µg/ml Ovomucoid protease inhibitor

The following protocol is used for preparative isolation of proteins that are expressed in the soluble or insoluble, whole cell fraction.

1. Grow and induce according to previously optimized conditions. Centrifuge 500 ml of culture at 5,000 x *g* for 5 min to pellet *E. coli* cells.

2. Resuspend the cells in 50 ml of *Extraction Buffer A*. Incubate at room temperature for 5 min. or until cells are lysed (sample should become viscous).

3. Add 5 ml of ***Extraction Buffer B***. Incubate at room temperature for 5 min or until no longer viscous.

4. Centrifuge at 25,000 x *g* for 1 hour at 4°C.

5. If your protein is expressed primarily in the soluble fraction, collect supernatant and apply to ANTI-FLAG M1 or M2 Affinity column. If expression is primarily in the insoluble, pellet fraction, solubilize with a detergent or chaotropic agent prior to affinity purification. The solubilizing agent should be compatible with the ANTI-FLAG M1 or M2 Affinity Gel.

IMMUNO-AFFINITY PURIFICATION OF FLAG FUSION PROTEINS

The choice of ANTI-FLAG M1 or M2 Affinity Gel for purification of a FLAG fusion protein is primarily dependent on the type of fusion protein. The ANTI-FLAG M1 Affinity Gel can be used for the purification of amino-terminal FLAG fusion proteins properly expressed by an ORF cloned into the pFLAG-ATS, pFLAG-1 or pFLAG-Shift$_{12}$ Expression Vectors. **(Table 1 and Figure 3).** The FLAG fusion protein can be eluted from the ANTI-FLAG M1 Affinity Gel using mild conditions with an agent which chelates calcium, such as EDTA or EGTA as well as by competition with FLAG peptide. Elution by Glycine-HCl (pH 3.5) is an option, but is the harshest of the possible elution conditions and your protein may not be resistant to acidic pH. The ANTI-FLAG M2 Affinity Gel can be used for the purification of amino-terminal or carboxy-terminal FLAG fusion proteins. **(Table 1 and Figure 3).** The FLAG fusion protein can be eluted from the ANTI-FLAG M2 Affinity Gel using mild conditions by competition with FLAG peptide or acid elution with Glycine-HCl (pH 3.5).

The choice of elution conditions used may also be dependent on the properties of your protein. EDTA is less specific than EGTA (chelates only calcium). EDTA is more effective than EGTA at elution of a FLAG fusion protein from the ANTI-FLAG M1 Affinity Gel. Elution can also be carried out using a buffer that lacks calcium. It is typically found that elution of FLAG fusion proteins by buffers which lack calcium occurs over more fractions than observed by elution using EDTA. Peptide elution may be preferred for purification of the FLAG fusion protein with the ANTI-FLAG M1 Affinity Gel if your protein requires a divalent cation which is chelated by EDTA or EGTA. Peptide elution is a mild, economical method that may be preferred over Glycine-HCl (pH 3.5) for purification of FLAG fusion proteins with the ANTI-FLAG M2 Affinity Gel.

The ANTI-FLAG M1 and M2 Affinity Gels have a theoretical binding capacity of approximately 40 nmol of a FLAG fusion protein/ml ANTI-FLAG Affinity Gel. They have been re-cycled in excess of 20 times under the appropriate conditions. It is best to reduce the quantity of affinity gel used for purification such that the FLAG fusion protein is loaded to saturation. In this way, non-specific binding of contaminating proteins to the column is held to a minimum. Flow rates using our columns in TBS buffer for the purification of the FLAG-BAP Positive Control Protein range from 0.5 - 1 ml/min. However, the viscosity, presence of particulate matter and column packing characteristics can dramatically reduce the flow rate. The presence of reducing agents in column loading buffers such as DTT or Mercaptoethanol can reduce the disulfide linkages between the heavy and light chains of the ANTI-FLAG Monoclonal Antibody. This may result in leaching of the ANTI-FLAG Monoclonal Antibody from the affinity gel and loss of binding capacity.

In batch mode, we recommend tumbling of the FLAG fusion protein with the gel overnight at 4°C at ≤ 5 column volumes of buffer. This is followed by centrifugation at 2,000 x *g* to pellet the gel beads for 5 min. Dilution with greater amounts of buffer may slow the binding of the FLAG fusion protein to the gel. However, these conditions can be optimized to improve recovery of your FLAG fusion protein.

Purification of FLAG Fusion Proteins with the ANTI-FLAG M1 Affinity Gel

Buffers:

TBS BUFFER
50 mM Tris-HCl (pH 7.4)
150 mM NaCl

TBS/Ca BUFFER
50 mM Tris-HCl (pH7.4)
150 mM NaCl
1 mM Calcium Chloride

TBS/EDTA
50 mM Tris-HCl (pH7.4)
150 mM NaCl
2 mM EDTA

TBS/A
50 mM Tris-HCl (pH7.4)
150 mM NaCl
0.02% Sodium Azide

Preequilibrate the column and all buffers to the temperature appropriate for purification of the protein of interest. Thermolabile proteins should be purified at 4°C. Photolabile proteins should be purified under conditions of reduced light intensity. Under most circumstances, purification at room temperature is acceptable. If there is a problem with proteases, perform column chromatography at +4°C. Cellular debris and particulate matter can clog the column and must, therefore, be removed. Highly viscous samples due to chromosomal DNA or RNA can also clog the column. These samples should be sonicated or nuclease treated to reduce viscosity. It may be useful to include an additional experiment with the FLAG-BAP Positive Control Protein at some point during purification.

The ANTI-FLAG M1 Affinity Gel is known to be resistant to the following detergents: ≤ 5.0% Tween-20; 5.0% Triton X-100; 0.1% NP-40; 0.1% CHAPS and 0.2% Digitonin. It can also be used with ≤ 1.0 M NaCl or 1.0 M Urea. Do not use the gel in the presence of SDS, Deoxycholate or Guanidine-HCl. This is by no means a comprehensive list.

Preparation of the Column
Note: *The following protocol is for a 1 ml column.*

1. Place the empty chromatography column on a firm support.

2. Remove the top and bottom tab and rinse the column twice with TBS. Allow buffer to drain from the column and leave residual TBS in the column to aid in packing the ANTI-FLAG M1 Affinity Gel in the next step.

Note: *If a drainage tube is placed on the column to control the flow, limit the length of tubing to under 25 cm.*

3. Thoroughly suspend the vial of ANTI-FLAG M1 Affinity Gel to make a homogeneous slurry of the gel beads. Immediately transfer the slurry to the column.

4. Allow the gel bed to drain and rinse the vial with TBS. Add the rinse to the column and allow to drain again.

Note: *The gel bed will not crack when excess solution is drained under normal circumstances, but do not let the gel bed dry.*

Washing the Column

1. Wash the gel by loading three sequential 5 ml aliquots of Glycine-HCl at pH 3.5, followed by 3 sequential 5 ml aliquots of TBS. Avoid disturbing the gel bed while loading. Let each aliquot drain completely before adding the next. Do not leave the column in Glycine-HCl for longer than 20 min.

2. The column is now ready for use.

Binding FLAG Fusion Proteins to the Column

1. Proper binding of FLAG fusion proteins to the ANTI-FLAG M1 Affinity column requires 0.15 M Sodium Chloride at pH 7.0 as well as the presence of calcium. Before loading the cell lysate or culture supernatant onto the ANTI-FLAG M1 Affinity column be sure that it contains at least 1mM $CaCl_2$.

Note: *If the sample contains particulate material, centrifuge or filter prior to applying to the column. Viscous samples should be treated with DNase or sonicated.*

2. Load the supernatant onto the column under gravity flow. Fill the column completely several times or attach the 12 ml column reservoir prior to loading for larger volumes. Depending on the protein and flow rate, all of the antigen may not bind. Multiple passes over the column will improve the binding efficiency.

3. Wash the column three times with 12 ml aliquots of TBS/Ca (TBS containing 1 mM $CaCl_2$).

Elution of FLAG Fusion Proteins by Acid Elution with Glycine

Elute the bound FLAG fusion protein from the column with 6 x 1 ml aliquots of 0.1M Glycine at pH, 3.5 into vials containing 15 - 25 µl of 1M Tris base at pH, 8.0. Do not leave the column in Glycine-HCl for longer than 20 min..

Elution of FLAG Fusion Proteins by Chelating Agent: EDTA

Incubate the column with 1 ml TBS/EDTA (TBS containing 2 mM EDTA) for 30 min to chelate calcium ions. Follow with 1 ml aliquots at 10 min intervals. Six elution aliquots are sufficient to elute the FLAG fusion protein.

Elution of FLAG Fusion Proteins by Competition with FLAG Peptide

Allow the column to drain completely. Elute the bound FLAG fusion protein by competitive elution with FLAG peptide.

FLAG-BAP (bacterial alkaline phosphatase) or the fusion protein of interest can be eluted with 5 column volumes of 100 µg/ml FLAG peptide (dissolved in TBS) applied sequentially in 5 x 1 ml fractions. Column packing quality, flow rate and specific properties of a FLAG fusion protein may influence the efficiency of peptide elution.

Storing the Column

Wash 3 times with 5 ml of TBS/A *(TBS containing 0.02% Sodium azide)* then add another 5 ml of TBS/A and store at 4°C without draining.

Recycling the Column

It is recommended that the column be regenerated immediately after use by washing with 3 x 5 ml aliquots of 0.1 M Glycine, HCl (pH 3.5). The column should be immediately re-equilibrated in TBS *(until the effluent is at neutral pH)*. With proper care the ANTI-FLAG M1 Affinity Gel column can be reused in excess of 20 times. However, the number of cycles observed will be dependent on variables such as sample condition.

Note: *Do not leave the column in 0.1 M Glycine-HCl for longer than 20 min.*

Purification of FLAG Fusion Proteins with the ANTI-FLAG M2 Affinity Gel

Pre-equilibrate the column, all buffers and perform all steps at room temperature. If there is a problem with proteases, perform column chromatography at + 4°C. Cellular debris and particulate matter can clog the column and must, therefore, be removed. Highly viscous samples due to chromosomal DNA or RNA can also clog the column. These samples should be sonicated or removed to reduce viscosity. It may be useful to include an additional experiment with a FLAG-BAP Positive Control Protein at some point in the experiment.

Preparation of the Column

1. Place the empty chromatography column on a firm support.

2. Remove the top and bottom tab and rinse the column twice with TBS. Allow buffer to drain from the column and leave residual TBS in the column to aid in packing the ANTI-FLAG M2 Affinity Gel in the next step.

Note: *If a drainage tube is placed on the column to control the flow, limit the length of tubing to under 25 cm.*

3. Thoroughly suspend the vial of ANTI-FLAG M2 Affinity Gel to make a homogeneous slurry of the gel beads. Immediately transfer the slurry to the column.

4. Allow the gel bed to drain and rinse the vial with TBS. Add the rinse to the column and allow to drain again.

Note: *The gel bed will not crack when excess solution is drained under normal circumstances, but do not to let the gel bed dry.*

Washing the Column

1. Wash the gel by loading three sequential 5 ml aliquots of 0.1 M Glycine-HCl (pH 3.5), followed by three sequential 5 ml aliquots of TBS. Avoid disturbing the gel bed while loading. Let each aliquot drain completely before adding the next.

2. The column is now ready for use.

Binding FLAG Fusion Proteins to the Column

1. Proper binding of FLAG fusion proteins to the ANTI-FLAG M2 Affinity Gel requires physiological ionic strength and pH. If the sample contains particulate material, centrifuge prior to applying to the column. Viscous samples should be sonicated or treated with DNase.

2. Load the sample onto the column under gravity flow. Fill the column completely several times for larger volumes. Depending upon the protein and flow rate, all of the antigen may not bind. Multiple passes over the column will improve the binding efficiency.

3. Wash the column three times with 12 ml aliquots of TBS.

Elution of FLAG Fusion Proteins by Acid Elution with Glycine

Elute the bound FLAG fusion protein from the column with 6 x 1 ml aliquots of 0.1 M Glycine at pH 3.5 into vials containing 15 - 25 µl of 1M Tris base at pH 8.0.

Elution of FLAG Fusion Proteins by Competition with FLAG Peptide

Same as for M1 Affinity Gel. See page 32.

Storing the Column

Wash 3 times with 5 ml of TBS/A *(TBS containing 0.02% Sodium azide)* then add another 5 ml of TBS/A and store at 4°C without draining.

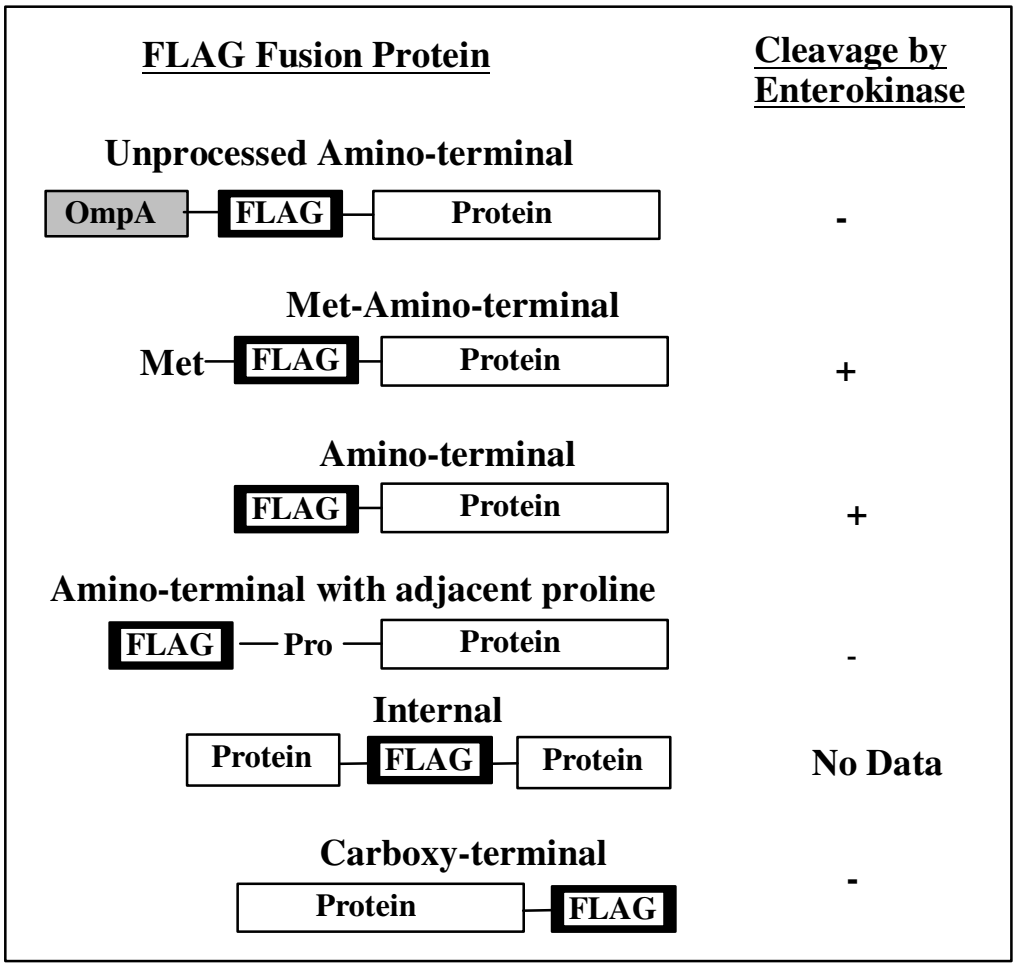
Recycling the Column

It is recommended that the column be regenerated immediately after use by washing with 3 x 5 ml aliquots of 0.1 M Glycine-HCl (pH 3.5) and immediately re-equilibrated in TBS (until the effluent is at neutral pH). With proper care the ANTI-FLAG M2 Affinity Gel column can be reused in excess of 20 times. However, the number of cycles observed will be dependent on variables such as sample condition.

Note: *Do not leave the column in 0.1 M Glycine-HCl for longer than 20 min.*

Figure 4.

Proteolytic Cleavage of FLAG Fusion Proteins by Enterokinase



-: indicates that FLAG fusion protein is not a substrate for enterokinase

+: indicates that FLAG fusion protein is a substrate for enterokinase

REMOVAL OF THE FLAG MARKER

The low molecular weight, hydrophilic FLAG marker should not interfere with the biological activity of the native protein. However, if removal of the FLAG peptide is required, using the protease enterokinase is usually effective. Enterokinase[39-42] recognizes the five amino acid sequence at the carboxy-terminus of the FLAG marker octapeptide. **(Figure 1)**. This binding site is very rare in nature, making it highly unlikely that enterokinase cleavage of a FLAG marker from a FLAG fusion protein will result in an internal cleavage of the native protein. A search of the entire NBRF Protein Database for the occurrence of this binding site shows its occurrence only in its natural substrate: trypsinogen and a few yeast proteins.

Enterokinase has a molecular weight of 150 kD. The enzyme is a heterodimer consisting of a heavy chain of molecular weight 115 kD and a light chain of 35 kD. The light and heavy chains are linked by 2 disulfide linkages. Enterokinase is also a glycoprotein containing 35% carbohydrate. Enterokinase will not cleave the FLAG peptide from a FLAG fusion protein if the first amino acid after the amino-terminal FLAG sequence is proline. It will also not cleave the FLAG sequence if it is located at the carboxy-terminus of the FLAG fusion protein or if the fusion protein still retains the OmpA signal peptide. **(Figure 4.)**

We find that ≥95% of 1µg of the FLAG-BAP fusion protein is cleaved with 1 unit of enterokinase in 18 hours at 37°C in enterokinase reaction buffer. However, this is not to be interpreted as our recommended conditions for cleavage of all FLAG fusion proteins. Cleavage by enterokinase is determined by the conversion of the 49 kD FLAG-BAP protein band to a 48 kD BAP protein band on a Coomassie Blue stained, 10% SDS-PAGE gel. Lack of protease activity is judged by the presence of no extraneous bands on the gel.

Optimization of digestion for other proteins may involve: **(1)** Variation of the incubation time (usually 2 - 18 hours); **(2)** Amount of enzyme and/or **(3)** Temperature of digestion.

Buffers:

ENTEROKINASE DILUTION BUFFER
10 mM Tris-HCl (pH 8)
10 mM Calcium Chloride

ENTEROKINASE REACTION BUFFER:
10 mM Tris -HCl
10 mM $CaCl_2$
Final buffer pH 8.0

1. Adjust the aliquot of FLAG fusion protein to a pH between 7.4 - 8.0.

2. Add 1.0 unit of enterokinase per µg of FLAG fusion protein. Digestion should be carried out in ***Enterokinase Reaction Buffer***. Mix and incubate the digestion mixture at 37°C.

3. Removal of the FLAG peptide can be assayed by a dot blot on nitrocellulose using the ANTI-FLAG M2 Monoclonal Antibody for N-terminal Met-FLAG peptide and ANTI-FLAG M1 Monoclonal Antibody for the N-terminal FLAG-peptide. The free peptide does not bind to nitrocellulose whereas the FLAG fusion protein does. Conversely, the peptide will bind to PVDF membrane which, therefore, cannot be used in a dot blot assay for proteolytic removal of FLAG peptides from FLAG fusion proteins. *(See following section for protocol)*.

It may be desirable to remove the FLAG peptide from the native protein following cleavage from the FLAG fusion protein. This may be possible by separation based on differences of molecular weight between the small FLAG peptide (1kD) and the protein of interest. This could include procedures such as gel filtration, dialysis or ultra-filtration.

It is sometimes desirable to remove enterokinase following digestion of the FLAG fusion protein. Although no one method is applicable for this purpose some properties of enterokinase may be useful towards this end. Removal of enterokinase following cleavage of the FLAG peptide is unnecessary if enterokinase-agarose (Recombinant Protein Cleavage Kit with Enterokinase Catalog No. RECOM-E) is used as the cleavage agent. Using this product, the agarose-bound enterokinase can be easily removed by centrifugation.

It is sometimes desirable to inhibit enterokinase activity following digestion of the FLAG fusion protein. Enterokinase is a serine protease that is known to be inhibited by serine protease inhibitors including soybean and pancreatic trypsin inhibitors. The pH activity profile of the enzyme indicates maximal activity between pH 6-10. The optimal temperature may vary with other protein substrates.

COLORIMETRIC DETECTION OF FLAG FUSION PROTEINS

FLAG fusion proteins can be detected during expression, affinity purification and enterokinase cleavage by dot, slot or Western blotting procedures. Detection on an immuno-blot is done using the ANTI-FLAG M1 or M2 Monoclonal Antibody as the primary probe. The ANTI-FLAG M2 Monoclonal Antibody is preferred for screening in most cases since it binds to FLAG fusion proteins with the FLAG marker at any location. **(Figure 3.)** The ANTI-FLAG M2 Monoclonal Antibody does not require $CaCl_2$, however the presence of $CaCl_2$ will not interfere with detection of FLAG fusion proteins. Blot detection of FLAG fusion proteins is best performed on nitrocellulose or PVDF membranes. Nylon is not recommended for protein blotting. Removal of the FLAG peptide by enterokinase is easily monitored by dot blot analysis on nitrocellulose using the ANTI-FLAG M1 or M2 Monoclonal Antibody. The FLAG peptide will not bind to the membrane and, therefore, will not be detected on the blot. PVDF membranes are not recommended for this specific purpose since the FLAG peptide binds to this membrane along with the FLAG fusion protein. FLAG peptide removal can also be confirmed by SDS-PAGE analysis on 10% gels since the native protein is 1000 daltons less in molecular weight than the intact FLAG fusion protein.

The colorimetric or chemiluminescent substrate employed for detection of Western and dot blots will affect the sensitivity of detection of FLAG fusion proteins. The most commonly employed substrates and their relative order of sensitivity is:

Luminol > TMB > AEC > NBT/BCIP > 4-CN

If you are having problems with the detection of a FLAG fusion protein, you may need to consider another substrate.

Peroxidase probes are preferred over alkaline phosphatase probes since alkaline phosphatase occurs more widely in nature than peroxidase and is, therefore, more likely to contaminate protein samples.

FLAG-BAP Positive Control Proteins are provided as a positive control to assure the functional integrity of the ANTI-FLAG M1 or M2 Monoclonal Antibody, the secondary probe as well as the colorimetric or chemiluminescent detection system employed. It is also useful as a marker on electrophoresis gels and as a control for Western transfer efficiency.

Detection of FLAG Fusion Proteins with 4-Chloro Naphthol Substrate by Dot Blot

Reagents:

WASH BUFFER
50 mM Tris-HCl (pH 7.4)
150 mM NaCl

4-CN REAGENT 1
60 mg 4-CN
20 ml cold Methanol

4-CN REAGENT 2
60 µl 30% Hydrogen peroxide
100 ml TBS (pH 7.4)

1. Apply 1 µl of culture or enterokinase digestion mixture as a small dot onto nitrocellulose approximately 3-4 mm in diameter. Dry and reapply in the same dot if a larger volume is needed for detection.

Note: *E. coli cell culture supernatants need not be denatured prior to blocking and may be dotted directly onto nitrocellulose.*

2. Place membrane on to Whatman 3MM paper saturated in 0.2 N NaOH, 1% SDS for 1 min, transfer to Whatman 3MM paper saturated with 0.2 M NaH_2PO_4 for 1 min and then to Whatman 3MM paper saturated with 0.02 M NaH_2PO_4 for 1 min.

3. Remove excess moisture from the blot.

4. Wet the blotting membrane in TBS and block by incubating for 30 min at room temperature in TBS containing 3% bovine serum albumin or other suitable blocking agent on a shaker. Decant blocking solution and briefly rinse with TBS again.

5. Incubate with 10 µg/ml of ANTI-FLAG M1 or M2 Monoclonal Antibody solution in TBS for 30 min at room temperature. Remove and save the antibody solution at 4°C up to one week for re-use.

6. Wash the dot blot 3 times with 3 changes of TBS on a shaker for 5 min each.

7. Incubate with an appropriate anti-mouse IgG Peroxidase-conjugated antibody diluted in TBS for 1 hour at room temperature.

8. Wash as in step **6.**

9. Incubate dot blot for 10 - 30 min with a freshly prepared substrate solution of 4-Chloro Naphthol (4-CN) until color develops.

10. Rinse blot three times with distilled water and dry. Store in a dry, dark location at -70°C.

Detection of FLAG Fusion Proteins with 4-CN Substrate on a Western Blot

1. Perform SDS polyacrylamide gel electrophoresis[43] of the cell supernatants, extracts, or purified FLAG fusion protein.

2. Transfer the proteins to a nitrocellulose membrane[44].

3. Block the membrane with TBS containing 3% bovine serum albumin or other suitable blocking agent for 1 hour on a shaker at room temperature and remove the blocking solution.

4. Incubate blot with ANTI-FLAG M1 or M2 Monoclonal Antibody solution (10 µg/ml) in TBS for 1 hour at room temperature. Remove and save the antibody solution at 4°C up to one week for re-use.

5. Wash with TBS three times on the shaker for 5 min each changing the wash solution each time.

6. Incubate with suitable anti-mouse reagent such as goat anti-mouse Peroxidase conjugated antibody diluted in TBS for 1 hour at room temperature.

7. Repeat step **5.**

8. Incubate nitrocellulose with freshly prepared peroxidase substrate, 4-Chloro Napthol (4-CN), until color develops (10 - 30 min).

9. Rinse blot with distilled water and dry. Store in a dry, dark location at -70°C.

MAPS OF THE SIGMA *E. COLI* EXPRESSION VECTORS

This section contains the maps and descriptions of the various Sigma Expression Vectors.

 pFLAG-MAC Expression Vector for the Cytoplasmic Expression of Met-Amino-Terminal FLAG Fusion Proteins **(Figure 5).**

 pFLAG-ATS Expression Vector for the Periplasmic Expression of the Amino-Terminal FLAG Fusion Proteins **(Figure 6).**

 pFLAG-CTC Expression Vector for the Cytoplasmic Expression of the Carboxy-Terminal FLAG Fusion Proteins **(Figure 7).**

 pFLAG-CTS Expression Vector for the Periplasmic Expression of the Carboxy-Terminal FLAG Fusion Proteins **(Figure 8).**

 pFLAG-1 Expression Vector for the Periplasmic Expression of the Amino-Terminal FLAG Fusion Proteins **(Figure 9).**

 pFLAG-2 Expression Vector for the Cytoplasmic Expression of Met-Amino-Terminal FLAG Fusion Proteins **(Figure 10).**

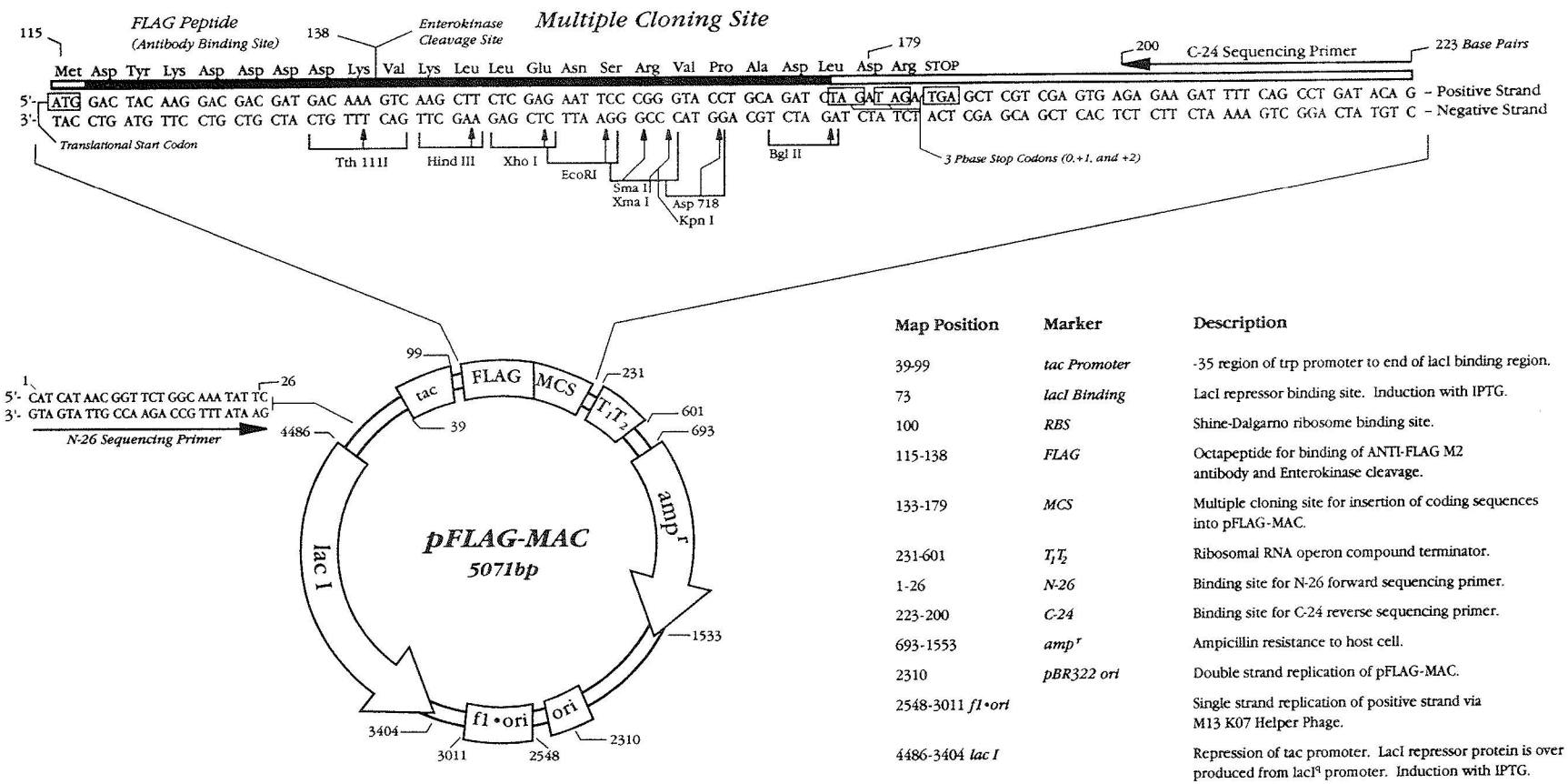
 pFLAG-Shift$_{12}$ Expression Vector for the Periplasmic Expression of the FLAG-Shift Proteins **(Figure 11).**

 pFLAG-Shift$_{12c}$ Expression Vector for the Cytoplasmic Expression of the FLAG-Shift Proteins **(Figure 12).**

 FLAG-BAP Positive Control Plasmids **(Figure 13).**

Figure 5.

Cytoplasmic Expression of Met-Amino-Terminal FLAG Fusion Proteins in *E. coli*



Map Position	Marker	Description
39-99	*tac Promoter*	-35 region of trp promoter to end of lacI binding region.
73	*lacI Binding*	LacI repressor binding site. Induction with IPTG.
100	*RBS*	Shine-Dalgarno ribosome binding site.
115-138	*FLAG*	Octapeptide for binding of ANTI-FLAG M2 antibody and Enterokinase cleavage.
133-179	*MCS*	Multiple cloning site for insertion of coding sequences into pFLAG-MAC.
231-601	T_1T_2	Ribosomal RNA operon compound terminator.
1-26	*N-26*	Binding site for N-26 forward sequencing primer.
223-200	*C-24*	Binding site for C-24 reverse sequencing primer.
693-1553	*amp r*	Ampicillin resistance to host cell.
2310	*pBR322 ori*	Double strand replication of pFLAG-MAC.
2548-3011 *f1•ori*		Single strand replication of positive strand via M13 K07 Helper Phage.
4486-3404 *lac I*		Repression of tac promoter. LacI repressor protein is over produced from $lacI^q$ promoter. Induction with IPTG.

Figure 6.

Periplasmic Expression of Amino-Terminal FLAG Fusion Proteins in *E. coli*

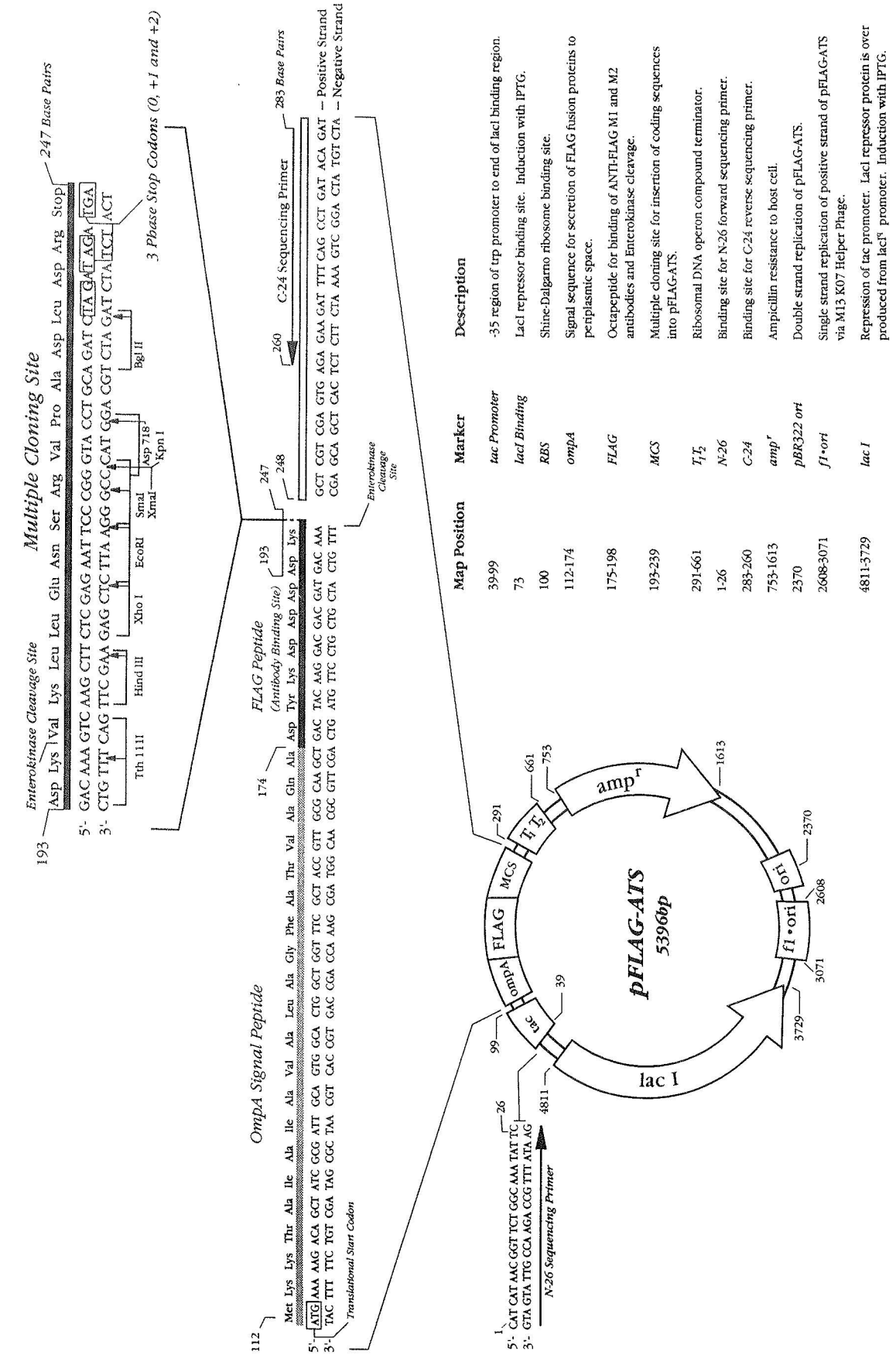
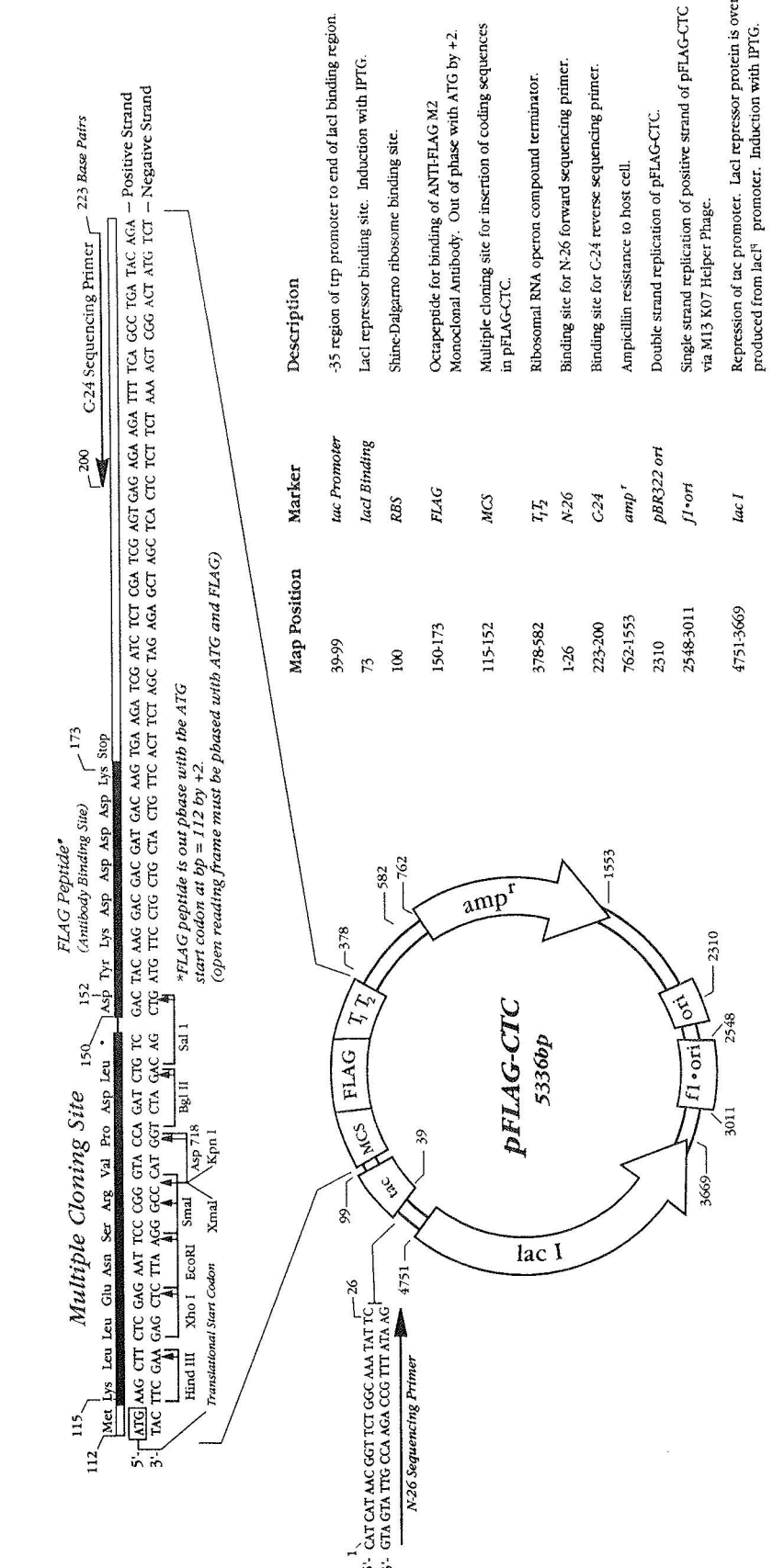


Figure 7.

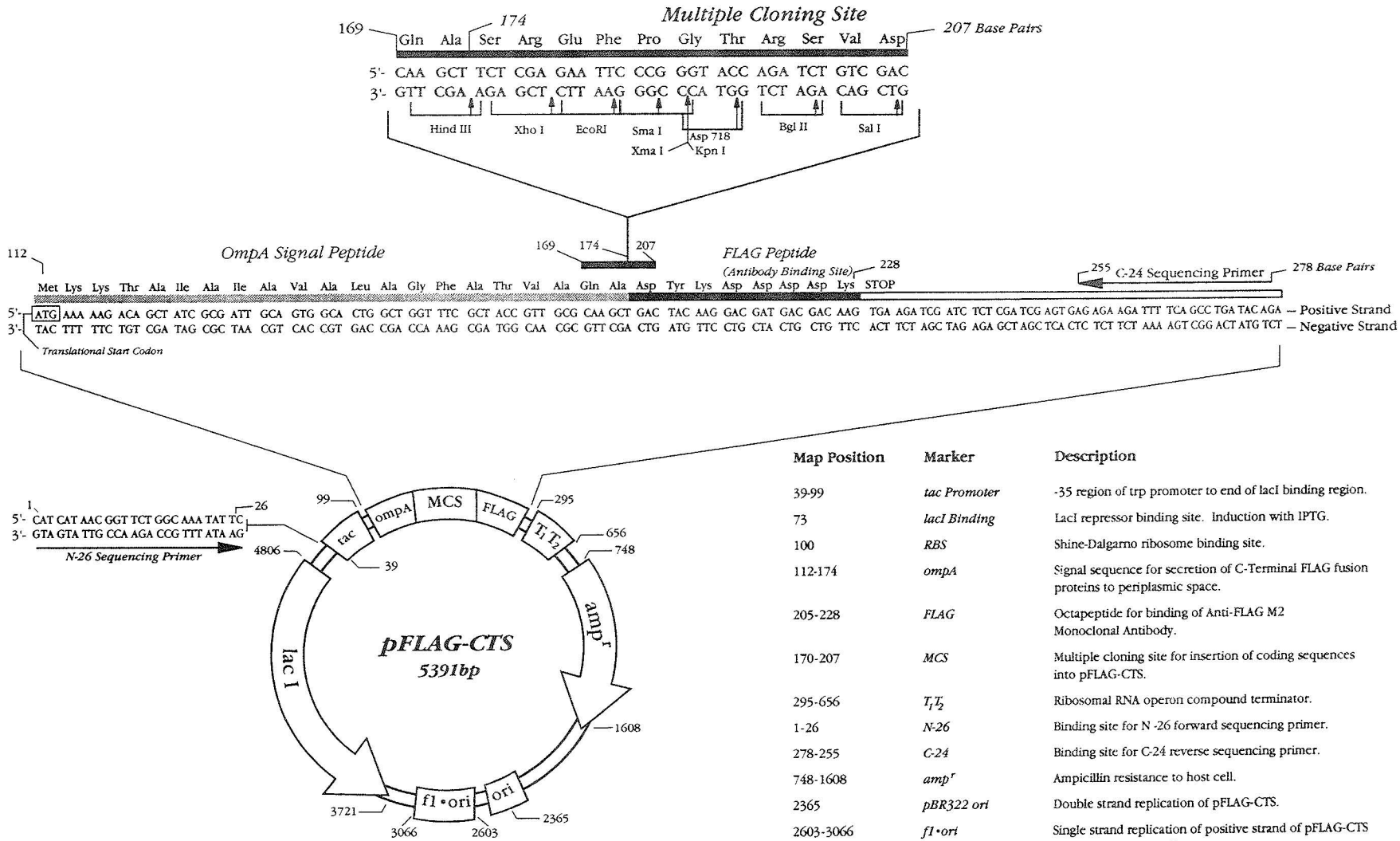
Cytoplasmic Expression of Carboxy-Terminal FLAG Fusion Proteins in *E. coli*



Map Position	Marker	Description
39-99	*tac Promoter*	-35 region of trp promoter to end of lacI binding region.
73	*lacI Binding*	LacI repressor binding site. Induction with IPTG.
100	*RBS*	Shine-Dalgarno ribosome binding site.
150-173	*FLAG*	Octapeptide for binding of ANTI-FLAG M2 Monoclonal Antibody. Out of phase with ATG by +2.
115-152	*MCS*	Multiple cloning site for insertion of coding sequences in pFLAG-CTC.
378-582	*T_1T_2*	Ribosomal RNA operon compound terminator.
1-26	*N-26*	Binding site for N-26 forward sequencing primer.
223-200	*C-24*	Binding site for C-24 reverse sequencing primer.
762-1553	*amp^r*	Ampicillin resistance to host cell.
2310	*pBR322 ori*	Double strand replication of pFLAG-CTC.
2548-3011	*f1•ori*	Single strand replication of positive strand of pFLAG-CTC via M13 K07 Helper Phage.
4751-3669	*lac I*	Repression of tac promoter. LacI repressor protein is over produced from $lacI^q$ promoter. Induction with IPTG.

Figure 8.

Periplasmic Expression of Carboxy-Terminal FLAG Fusion Proteins in *E. coli*

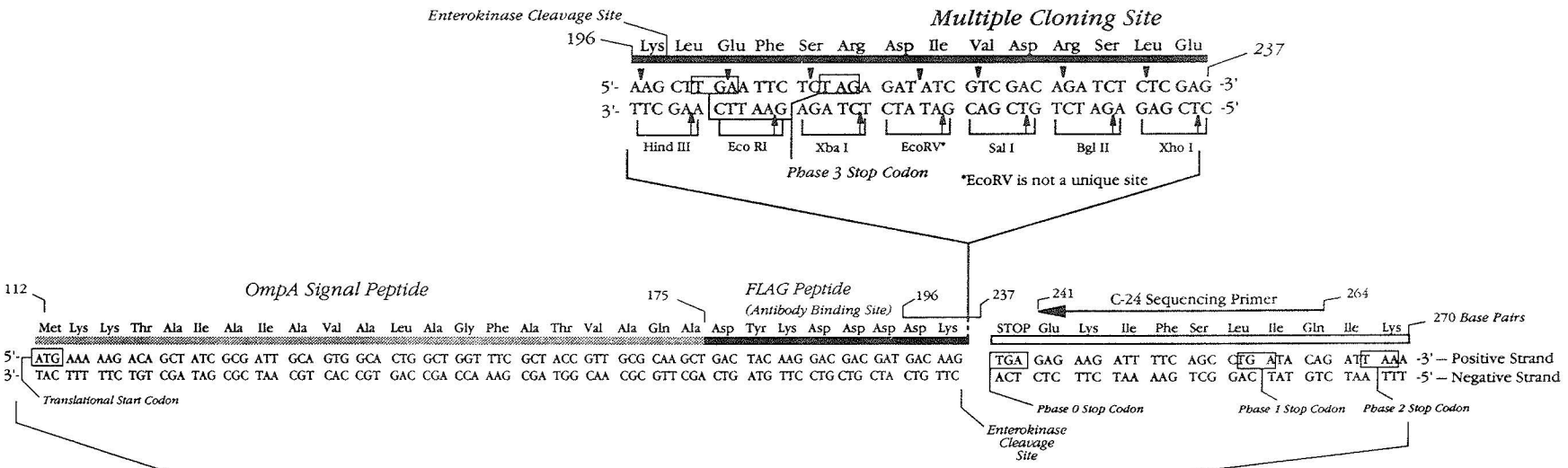


Map Position	Marker	Description
39-99	*tac Promoter*	-35 region of trp promoter to end of lacI binding region.
73	*lacI Binding*	LacI repressor binding site. Induction with IPTG.
100	*RBS*	Shine-Dalgarno ribosome binding site.
112-174	*ompA*	Signal sequence for secretion of C-Terminal FLAG fusion proteins to periplasmic space.
205-228	*FLAG*	Octapeptide for binding of Anti-FLAG M2 Monoclonal Antibody.
170-207	*MCS*	Multiple cloning site for insertion of coding sequences into pFLAG-CTS.
295-656	T_1T_2	Ribosomal RNA operon compound terminator.
1-26	*N-26*	Binding site for N -26 forward sequencing primer.
278-255	*C-24*	Binding site for C-24 reverse sequencing primer.
748-1608	*amp^r*	Ampicillin resistance to host cell.
2365	*pBR322 ori*	Double strand replication of pFLAG-CTS.
2603-3066	*f1•ori*	Single strand replication of positive strand of pFLAG-CTS via M13 K07 Helper Phage.
4806-3721	*lac I*	Repression of tac promoter. LacI repressor protein is over produced from lacI^q promoter. Induction with IPTG.

Catalog No. E5269

Figure 9.

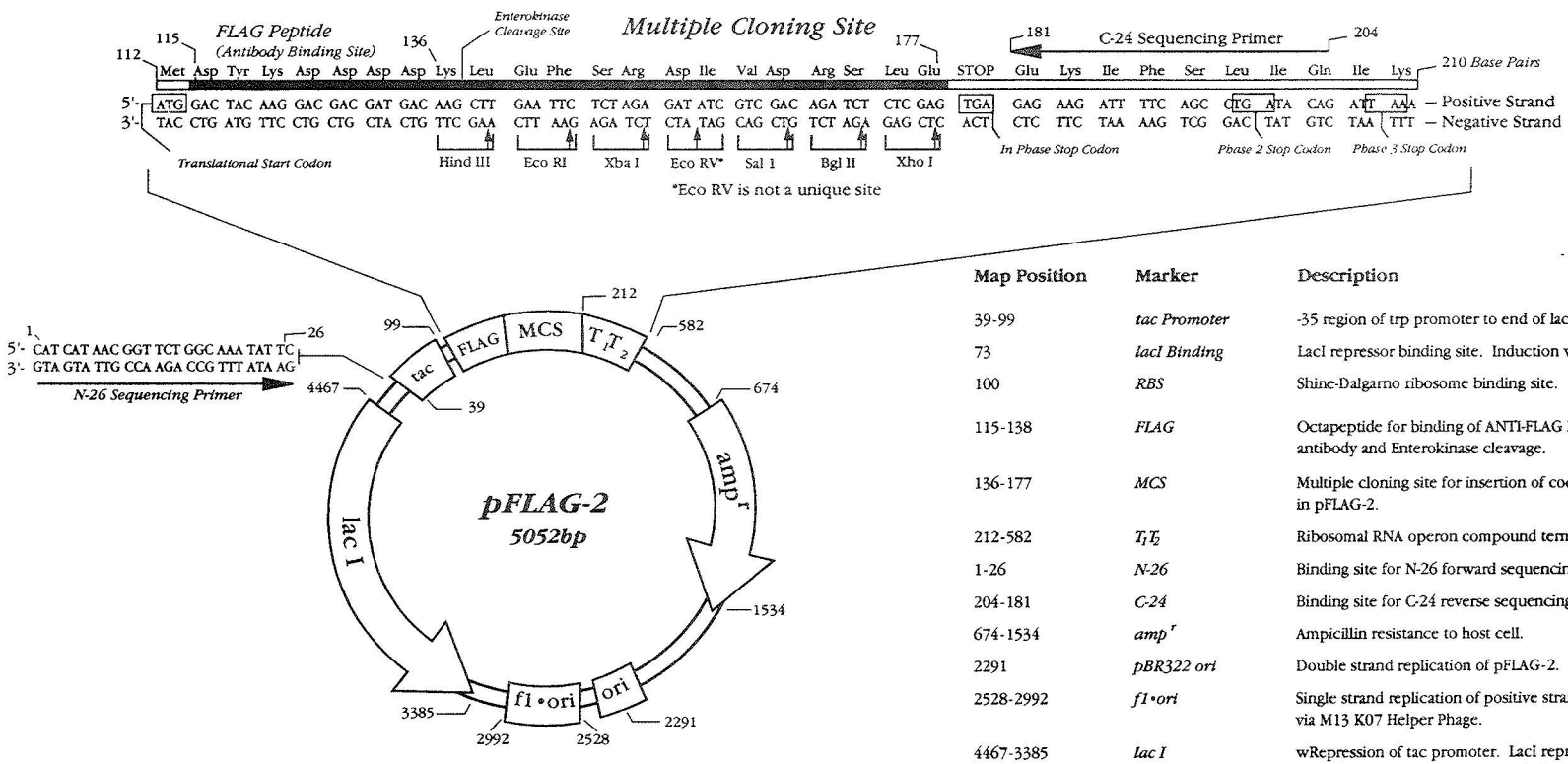
Periplasmic Expression of Amino-Terminal FLAG Fusion Proteins in *E. coli*



Map Position	Marker	Description
39-99	*tac Promoter*	-35 region of trp promoter to end of lacI binding region.
73	*lacI Binding*	LacI repressor binding site. Induction with IPTG.
100	*RBS*	Shine-Dalgarno ribosome binding site.
112-174	*ompA*	Signal sequence for secretion of FLAG fusion proteins to periplasmic space.
175-198	*FLAG*	Octapeptide for binding of Anti-FLAG antibodies and Enterokinase cleavage.
196-237	*MCS*	Multiple cloning site for insertion of coding sequences into pFLAG-1.
272-642	T_1T_2	Ribosomal RNA operon compound terminator.
1-26	*N-26*	Binding site for N-26 forward sequencing primer.
264-241	*C-24*	Binding site for C-24 reverse sequencing primer.
734-1594	*amp^r*	Ampicillin resistance to host cell.
2351	*pBR322 ori*	Double strand replication of pFLAG-1.
2589-3052	*f1•ori*	Single strand replication of positive strand via M13 K07 Helper Phage.
4792-3710	*lac I*	Repression of tac promoter. LacI repressor protein is over produced from lacI^q promoter. Induction with IPTG.

Figure 10.

Cytoplasmic Expression of Met-Amino-Terminal FLAG Fusion Proteins in *E. coli*

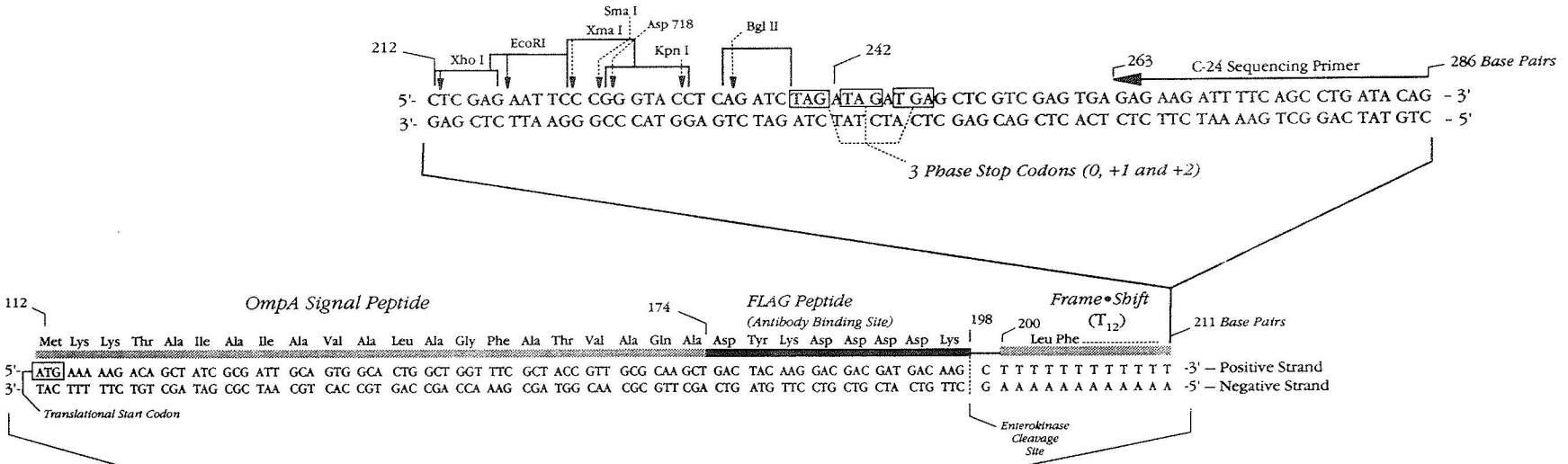


Map Position	Marker	Description
39-99	*tac Promoter*	-35 region of trp promoter to end of lacI binding region.
73	*lacI Binding*	LacI repressor binding site. Induction with IPTG.
100	*RBS*	Shine-Dalgarno ribosome binding site.
115-138	*FLAG*	Octapeptide for binding of ANTI-FLAG M2 antibody and Enterokinase cleavage.
136-177	*MCS*	Multiple cloning site for insertion of coding sequences in pFLAG-2.
212-582	*T_1T_2*	Ribosomal RNA operon compound terminator.
1-26	*N-26*	Binding site for N-26 forward sequencing primer.
204-181	*C-24*	Binding site for C-24 reverse sequencing primer.
674-1534	*amp^r*	Ampicillin resistance to host cell.
2291	*pBR322 ori*	Double strand replication of pFLAG-2.
2528-2992	*f1•ori*	Single strand replication of positive strand via M13 K07 Helper Phage.
4467-3385	*lac I*	wRepression of tac promoter. LacI repressor protein is over produced from lacI^q promoter. Induction with IPTG.

Figure 11.

Periplasmic Expression of Amino-Terminal FLAG-SHIFT Fusion Proteins in *E. coli*

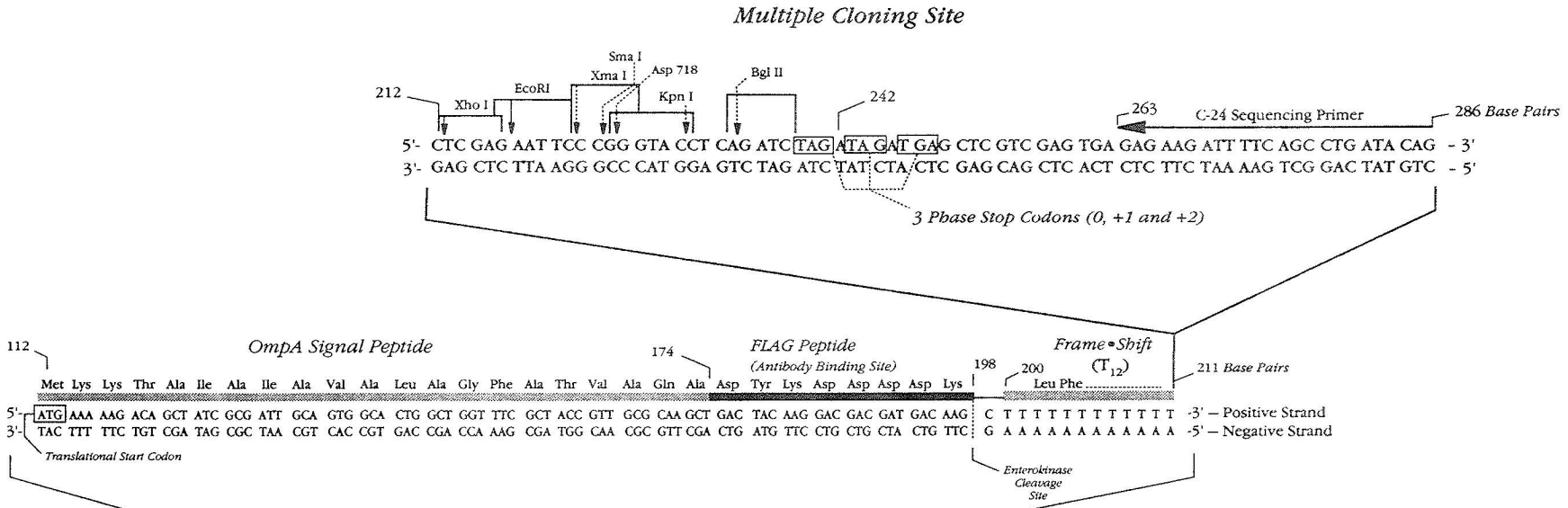
Multiple Cloning Site



Map Position	Marker	Description
1-26	N-26	Binding site for N-26 Sequencing Primer
39-99	*tac Promoter*	-35 region of trp promoter to end of lacI binding region.
73	*lacI Binding*	LacI repressor binding site. Induction with IPTG.
100	*RBS*	Shine-Dalgarno ribosome binding site.
112-174	*ompA*	Signal sequence for secretion of FLAG fusion proteins to periplasmic space.
175-198	*FLAG*	Octapeptide for binding of ANTI-FLAG M1 or M2 Monoclonal Antibody and Enterokinase cleavage.
200-211	*SHIFT*	Translational Reading Frameshift sequence for expression of an ORF in all 3 reading frames.
212-242	*MCS*	Multiple cloning site.
294-664	T_1T_2	Ribosomal RNA operon compound terminator.
286-263	*C-24*	Binding site for C-24 reverse sequencing primer.
756-1616	*amp*^{r}	Ampicillin resistance to host cell.
2373	*pBR322 ori*	Double strand replication of pFLAG•SHIFT$_{12}$.
2611-3074	*f1•ori*	Single strand replication of positive strand via M13 K07 Helper Phage.
4549-3467	*lac I*	Repression of tac promoter. LacI repressor protein is over produced from lacI^q promoter. Derepression with IPTG.

Figure 12.

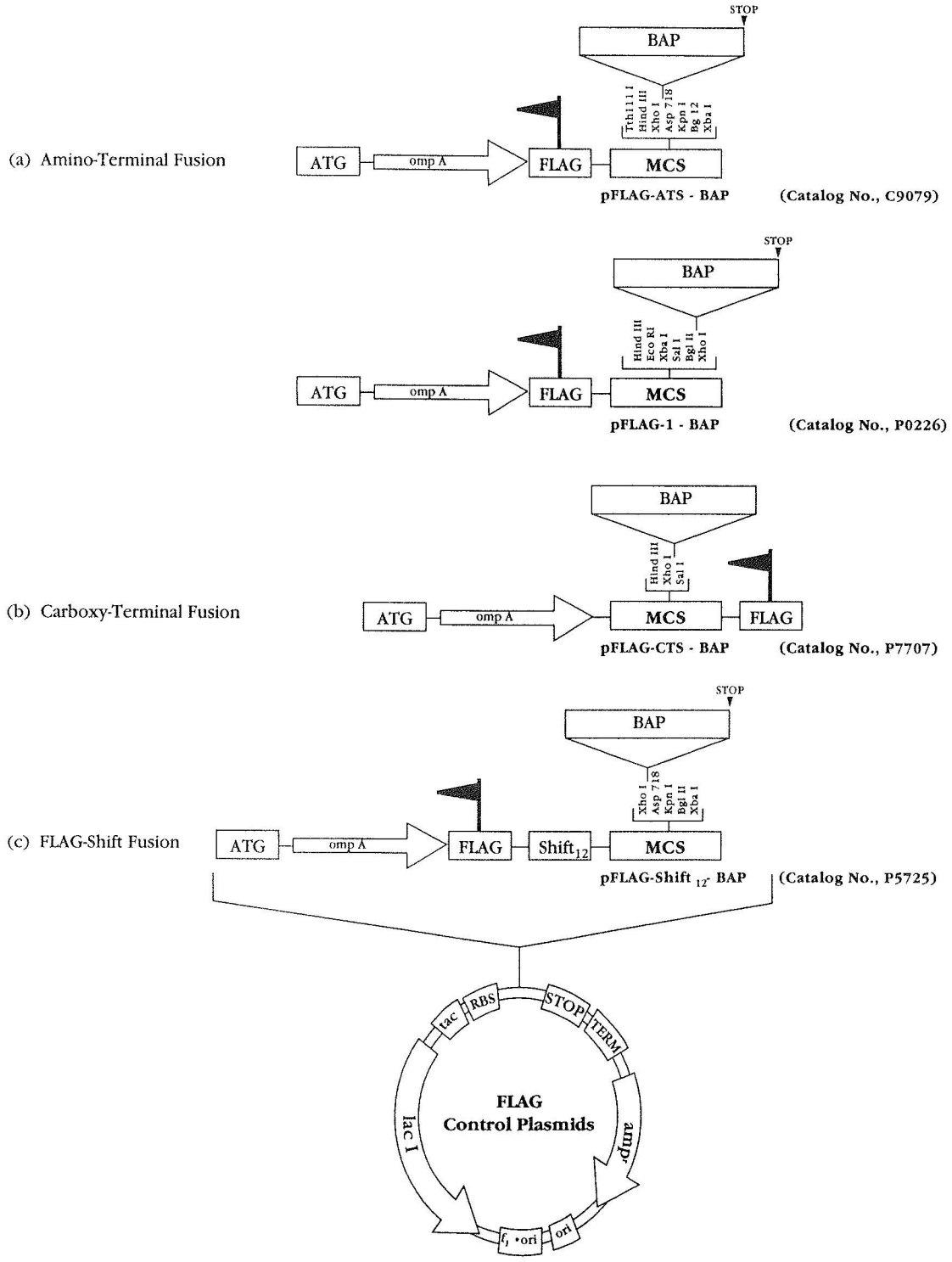
Cytoplasmic Expression of Amino-Terminal FLAG-SHIFT Fusion Proteins in *E. coli*



Map Position	Marker	Description
1-26	N-26	Binding site for N-26 Sequencing Primer
39-99	*tac Promoter*	-35 region of trp promoter to end of lacI binding region.
73	*lacI Binding*	LacI repressor binding site. Induction with IPTG.
100	*RBS*	Shine-Dalgarno ribosome binding site.
112-174	*ompA*	Signal sequence for secretion of FLAG fusion proteins to periplasmic space.
175-198	*FLAG*	Octapeptide for binding of ANTI-FLAG M1 or M2 Monoclonal Antibody and Enterokinase cleavage.
200-211	*SHIFT*	Translational Reading Frameshift sequence for expression of an ORF in all 3 reading frames.
212-242	*MCS*	Multiple cloning site.
294-664	T_1T_2	Ribosomal RNA operon compound terminator.
286-263	*C-24*	Binding site for C-24 reverse sequencing primer.
756-1616	*amp*^{r}	Ampicillin resistance to host cell.
2373	*pBR322 ori*	Double strand replication of pFLAG•SHIFT$_{12}$.
2611-3074	*f1•ori*	Single strand replication of positive strand via M13 K07 Helper Phage.
4549-3467	*lac I*	Repression of tac promoter. LacI repressor protein is over produced from lacI^q promoter. Derepression with IPTG.

Figure 13.

FLAG-BAP Positive Control Plasmids



The FLAG-BAP Positive Control Plasmids are useful positive controls for protein expression, immunological detection and immunological detection and immuno-affinity purification of FLAG fusion proteins. ▼STOP = Translational stop codon.

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Product Number	Description	Package Size
Kits		
FL-MA FL-MC	Mammalian Amino-terminal FLAG Expression Kit Mammalian Carboxy-terminal FLAG Expression Kit	1 kit
FL-A FL-C FL-S	Bacterial Amino-terminal FLAG Expression Kit Bacterial Carboxy-terminal FLAG Expression Kit Bacterial FLAG®-Shift Expression Kit	1 kit
FL-YA	Yeast Amino-terminal FLAG Expression Kit	1 kit
Mammalian expression system components		
E 7273 E 7398 E 7523 E 7648 E 7773	pFLAG-CMV-1 expression vector (for section) pFLAG-CMV-2 expression vector (for cytoplasmic expression) pFLAG-CMV-5a expression vector (for intra- or extracellular expression) pFLAG-CMV-5b expression vector (for intra- or extracellular expression) pFLAG-CMV-5c expression vector (for intra- or extracellular expression)	10 µg
P 4975 P 5100 P 5225	pFLAG-CMV-1-BAP positive control plasmid pFLAG-CMV-2-BAP positive control plasmid pFLAG-CMV-5b-BAP positive control plasmid	20 µg
P 5350 P 5475	C-CMV-24 sequencing primer (24-mer) N-CMV-30 sequencing primer (30-mer)	1 µg
Bacterial expression system components		
E 5769 E 5644 E 5269 E 5394	pFLAG-ATS expression vector (for periplasmic expression) pFLAG-MAC expression vector (for cytoplasmic expression) pFLAG-CTS expression vector (for periplasmic expression) pFLAG-CTC expression vector (for cytoplasmic expression)	10 µg
E 8023 E 7898	FLAG-Shift$_{12}$ expression vector (for periplasmic expression) FLAG-Shift$_{12c}$ expression vector (for cytoplasmic expression)	10 µg
C 9079 P 7707	pFLAG-ATS-BAP positive control plasmid pFLAG-CTS-BAP positive control plasmid	1 µg
P 5725	FLAG-Shift$_{12}$-BAP positive control plasmid	1 µg
P 7832 P 7957	N-26 sequencing primer (26-mer) C-24 sequencing primer (24-mer)	1 µg
Yeast expression system components		
Y 4875	Yeast host strain BJ3505	100 µl
E 9020	YEpFLAG-1 expression vector (for expression and secretion)	10 µg
C 2082	YEpFLAG-1-BAP positive control plasmid	10 µg
P 8959 P 8834	YαN-21 sequencing primer (21-mer) YcC-21 sequencing primer (21-mer)	1 µg
Common FLAG components		
F 3040 F 3165 F 4042	ANTI-FLAG M1 monoclonal antibody ANTI-FLAG M2 monoclonal antibody ANTI-FLAG M5 monoclonal antibody	200 µg 1 mg 5 mg
A 1080 A 2220	ANTI-FLAG M1-agarose affinity resin ANTI-FLAG M2-agarose affinity resin	1 ml 5 ml 10 ml 25 ml
F 9291	ANTI-FLAG-BioM2 antibody-biotin conjugate	200 µg 1 mg 5x1 mg
F 2922	ANTI-FLAG-BioM5 antibody-biotin conjugate	200 µg 1 mg
F 3290	FLAG peptide (8 a.a., 1013 Da)	4 mg 25 mg
P 7582 P 7457 P 5975	N-terminal FLAG-BAP control protein C-terminal BAP-FLAG control protein Met-FLAG-BAP control protein	100 µg
E 5144	Enterokinase	50 units 250 units

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