VariFlex Bacterial Protein Expression System

Instruction Manual

Catalog #240162 (N-terminal SBP Vector Set)
#240163 (N-terminal SBP Vector and Purification Kit)
#240164 (N-terminal SBP-SET Vector Set)
#240165 (N-terminal SBP-SET Vector and Purification Kit)
#240172 (N-terminal SET Vector Set)
#240174 (C-terminal SBP Vector)
#240175 (C-terminal SBP Vector and Purification Kit)
#240176 (C-terminal SBP-SET Vector Set)
#240177 (C-terminal SBP-SET Vector and Purification Kit)
#240184 (C-terminal SET Vector Set)

Revision B.0

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240162-12

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VariFlex Bacterial Protein Expression System

CONTENTS

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MATERIALS PROVIDED

N-terminal SBP Vector Set (Catalog #240162)

Component	Concentration	Quantity
pBEn-SBPa vector	1 µg/µl	20 µg
pBEn-SBPb vector	1 µg/µl	20 µg
pBEn-SBPc vector	1 µg/µl	20 µg

N-terminal SBP Vector and Purification Kit (Catalog #240163)

Component	Concentration	Quantity
N-terminal SBP Vector Set (Catalog #240162)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml

N-terminal SBP-SET Vector Set (Catalog #240164)

Component	Concentration	Quantity
pBEn-SBP-SET1a vector	1 µg/µl	20 µg
pBEn-SBP-SET1b vector	1 µg/µl	20 µg
pBEn-SBP-SET1c vector	1 µg/µl	20 µg
pBEn-SBP-SET2a vector	1 µg/µl	20 µg
pBEn-SBP-SET2b vector	1 µg/µl	20 µg
pBEn-SBP-SET2c vector	1 µg/µl	20 µg
pBEn-SBP-SET3a vector	1 µg/µl	20 µg
pBEn-SBP-SET3b vector	1 µg/µl	20 µg
pBEn-SBP-SET3c vector	1 µg/µl	20 µg

N-terminal SBP-SET Vector and Purification Kit (Catalog #240165)

Component	Concentration	Quantity
N-terminal SBP-SET Vector Set (Catalog #240164)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml

N-terminal SET Vector Set (Catalog #240172)

Component	Concentration	Quantity
pBEn-SET1a vector	1 µg/µl	20 µg
pBEn-SET1b vector	1 µg/µl	20 µg
pBEn-SET1c vector	1 µg/µl	20 µg
pBEn-SET2a vector	1 µg/µl	20 µg
pBEn-SET2b vector	1 µg/µl	20 µg
pBEn-SET2c vector	1 µg/µl	20 µg
pBEn-SET3a vector	1 µg/µl	20 µg
pBEn-SET3b vector	1 µg/µl	20 µg
pBEn-SET3c vector	1 µg/µl	20 µg

Revision B.0

C-terminal SBP Vector (Catalog #240174)

Component	Concentration	Quantity
pBEc-SBP vector	1 µg/µl	20 µg

C-terminal SBP Vector and Purification Kit (Catalog #240175)

Component	Concentration	Quantity
C-terminal SBP Vector (Catalog #240174)	—	20 µg
Streptavidin resin	—	1.25 ml

C-terminal SBP-SET Vector Set (Catalog #240176)

Component	Concentration	Quantity
pBEc-SBP-SET1 vector	1 µg/µl	20 µg
pBEc-SBP-SET2 vector	1 µg/µl	20 µg
pBEc-SBP-SET3 vector	1 µg/µl	20 µg

C-terminal SBP-SET Vector and Purification Kit (Catalog #240177)

Component	Concentration	Quantity
C-terminal SBP-SET Vector Set (Catalog #240176)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml

C-terminal SET Vector Set (Catalog #240184)

Component	Concentration	Quantity
pBEc-SET1 vector	1 µg/µl	20 µg
pBEc-SET2 vector	1 µg/µl	20 µg
pBEc-SET3 vector	1 µg/µl	20 µg

STORAGE CONDITIONS

Streptavidin Resin: 4°C
All Other Components: –20°C

ADDITIONAL MATERIALS REQUIRED

T4 DNA ligase
Ligase buffer[§]
TE buffer[§]
14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
BL21-Gold (DE3) competent cells
Thrombin

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

NOTICES TO PURCHASER

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INTRODUCTION

The Agilent VariFlex bacterial protein expression system is a series of pET-based vectors that offer solutions to challenges in protein expression and enhance the utility of *E. coli* as an expression host. Available tags include three different solubility enhancement tags (SETs) which are designed to increase protein solubility, and the streptavidin binding peptide (SBP) purification tag. The VariFlex vectors are available with one or two tags in various combinations. Figure 1 shows the amino acid sequences of each tag.

One of the most difficult problems in expressing eukaryotic genes in bacterial systems is the lack of solubility of the expression product. Often times, expression in a more time-consuming, lower-yielding, expensive host such as yeast, insect, or mammalian cells is necessary. To address this issue, the VariFlex SET tags increase the solubility of many problem proteins in *E. coli*. Although the mechanism by which the SET tags improve solubility has not yet been confirmed, the tags are thought to enhance solubility of the fusion protein by providing a net negative charge, which is thought to prevent aggregation and provide more time for correct protein folding in vivo.[1] The SET tags are based on the C-terminal portion of the T7 phage gene 10B sequence (T7B) which has a net charge of –6. The SET1 tag is the wild-type T7B sequence, while the SET2 and SET3 tags are mutants of T7B that further increase the net negative charge to –12 and –18 respectively. Since every protein is unique, the optimal SET tag needs to be determined empirically for each protein of interest. We therefore offers the SET-tagged vectors as complete sets, where vectors containing each of the three SET tag variants are provided.

In addition to the SET tags, Agilent offers vectors containing the SBP tag, which provides a method for efficient purification of the protein of interest. The SBP tag, a synthetic sequence isolated from a random peptide library, has a high affinity for streptavidin resin (~2×10^{-9} M), and can be effectively eluted from the resin with biotin.[2, 3] The SBP tag has a low positive net charge (+1), making it an ideal purification tag when combined with the SET tags, since its effect on the SET tag negative charge is minimal.

```
SET1 Tag   MDPEEASVTSTEETLTPAQEAARTRAANKARKEAELAAATAEQ

SET2 Tag   MDPEEASVTSTEETLTPAQEAAETEAANKARKEAELEAETAEQ
                                 * *           * *

SET3 Tag   MDPEEASVTSTEETLTPAQEAAETEAANKAEEEAELEAETAEQ
                                 * *     **    * *

SBP Tag    MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSGGCKLG
```

FIGURE 1 Amino acid sequences of the solubility enhancement tags (SET1, SET2, and SET3), and the streptavidin binding peptide (SBP) tag. The asterisks indicate mutations present in the variants of the SET tag.

THE VARIFLEX PROTEIN EXPRESSION VECTORS

The VariFlex protein expression vectors are derived from the pET-11 vector series (see Figures 2, 3). The vectors are engineered to take advantage of the features of the bacteriophage T7 gene *10* promoter and leader sequence that allow high selectivity of the promoter by T7 RNA polymerase, tight repression in the uninduced state, and high-level expression upon induction.[4, 5] The VariFlex vectors use the T7 *lac* promoter configuration and carry a copy of the *lacI* gene to mediate this tight repression.

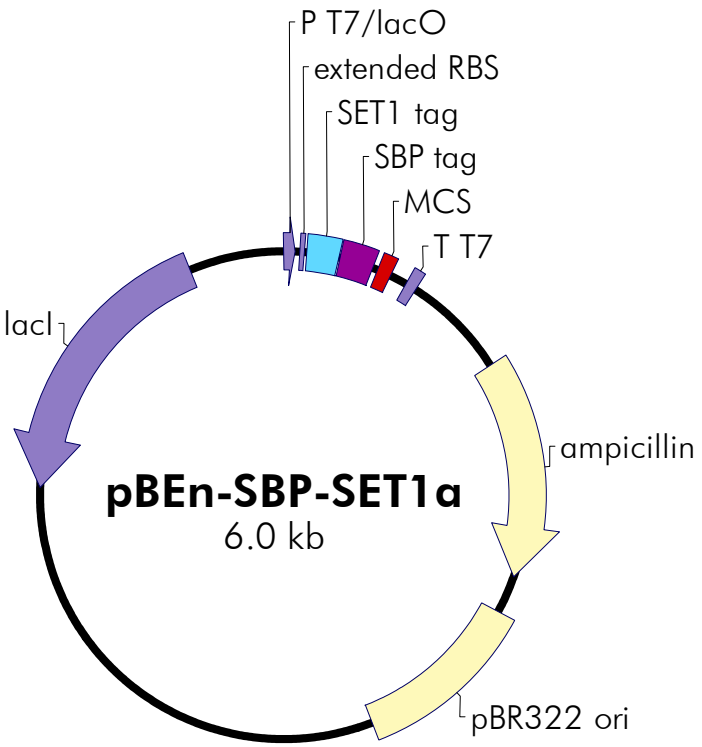
Each VariFlex vector carries one or two tags in different combinations, providing flexibility depending on the desired applications. These tags include the solubility enhancement tags 1–3 and the streptavidin binding peptide for protein purification. The tags are available as fusions to either the N- or C-terminus of the protein of interest, and the N-terminal vectors are provided in all three reading frames. A thrombin protease cleavage site is present between the tag(s) and the cloning site so the tags can be easily removed for further protein analysis.

The pBEn vectors are based on the pET-11a vector and contain the tag coding sequence(s) inserted upstream of a multiple cloning site (MCS) to allow for fusion of the tag(s) at the N-terminus of the cloned protein-coding sequence. The efficient translation of the tags in *E. coli* ensures that fusion proteins containing the tags at the N terminus will be consistently expressed at high levels. The recognition sequence for thrombin is inserted between the tag coding sequences and the MCS. Digestion of purified fusion protein with thrombin occurs between the arginine and glycine residues within the thrombin recognition sequence.

The pBEc vectors are based on the pET-11d vector and contain the tag coding sequence(s) inserted downstream of the cloning site to allow for fusion of the tag(s) at the C-terminus of the cloned protein-coding sequence. Inserts are cloned between the *Nco* I site, which contains an ATG positioned for optimal translation from the T7 gene *10* ribosome-binding site (RBS), and the *Bam*H I site. Alternatively, inserts can be cloned between the *Nhe* I and *Bam*H I sites. Thrombin digestion of proteins expressed from the pBEc vectors result in the retention of the four N-terminal amino acids (MYPR) from the thrombin recognition sequence.

Caution *The T7 gene 10 leader and the C-terminal fusion tags, beginning with the Gly-Ser residues encoded by the* Bam*H I restriction site, are in* ***separate frames****. Although bi-directional cloning is not recommended, if cloning into the* Bam*H I restriction site, care should be taken that the protein coding sequence of interest is fused in frame with both the T7 gene 10 leader and the C-terminal fusion tag. If cloning bi-directionally into* Nco *I or* Nhe *I, the inserted amino acid sequence should be in frame with the C-terminal fusion tag beginning with the Gly-Ser residues encoded by the* Bam*H I site.*

pBEn-SBP-SET1a Vector Map



pBEn-SBP-SET1a Multiple Cloning Site Region
sequence shown (323–433)

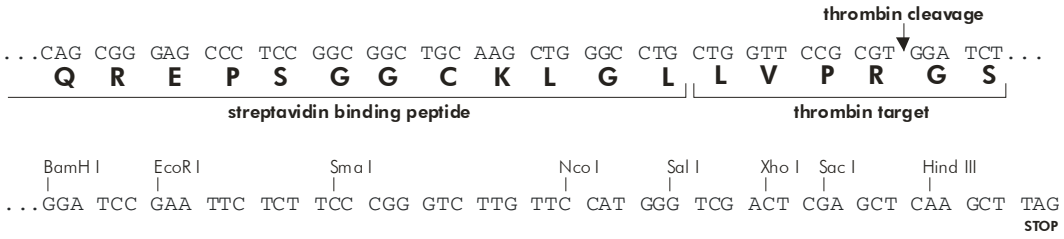
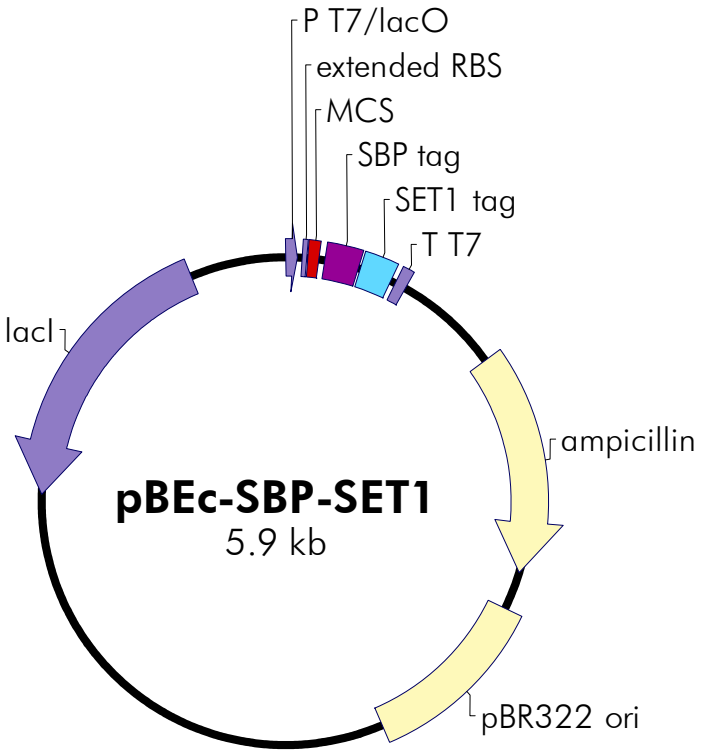


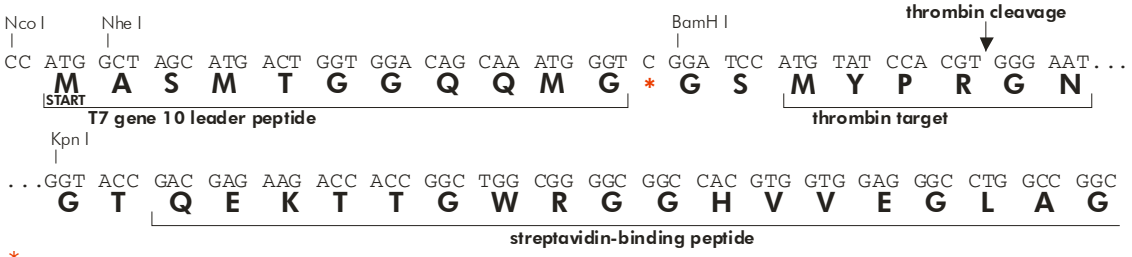
FIGURE 2 Vector map and multiple cloning region of the pBEn-SBP-SET1a bacterial protein expression vector. See Table I for vector feature locations. This figure is intended to be a general representation of the N-terminal vectors. Other vectors may include different tag combinations, reading frames, and restriction sites. For specific vector information, please refer to *www.genomics.agilent.com/vectorMapsAndSequence.jsp*.

pBEc-SBP-SET1 Vector Map



pBEc-SBP-SET1 Multiple Cloning Site Region

sequence shown (87–206)



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

FIGURE 3 Vector map and multiple cloning region of the pBEc-SBP-SET1 bacterial protein expression vector. See Table I for vector feature locations. This figure is intended to be a general representation of the C-terminal vectors. Other vectors may include different tag combinations, reading frames, and restriction sites. For specific vector information, please refer to www.genomics.agilent.com/vectorMapsAndSequence.jsp.

TABLE I

Features of the Bacterial Protein Expression Vectors

	T7 promoter with *lac* operon	extended ribosome binding site	solubility enhancement tag 1 (SET1 tag)	solubility enhancement tag 2 (SET2 tag)	solubility enhancement tag 3 (SET3 tag)	streptavidin binding peptide (SBP tag)	multiple cloning site	T7 terminator	ampicillin resistance (*bla*) ORF	pBR322 origin of replication	*lacI* repressor ORF
pBEn-SBPa	1–44	64–80	—	—	—	92–223	245–299	369–411	832–1689	1840–2507	4387–5475
pBEn-SBPb	1–44	64–80	—	—	—	92–223	246–300	370–412	883–1690	1841–2508	4388–5476
pBEn-SBPc	1–44	64–80	—	—	—	92–223	247–301	371–413	834–1691	1842–2509	4389–5477
pBEn-SBP-SET1a	1–44	64–80	92–217	—	—	224–355	377–431	501–543	964–1821	1972–2639	4519–5607
pBEn-SBP-SET1b	1–44	64–80	92–217	—	—	224–355	378–432	502–544	965–1812	1973–2640	4520–5608
pBEn-SBP-SET1c	1–44	64–80	92–217	—	—	224–355	379–433	503–545	966–1823	1974–2641	4521–5609
pBEn-SBP-SET2a	1–44	64–80	—	92–217	—	224–355	377–431	501–543	964–1821	1972–2639	4519–5607
pBEn-SBP-SET2b	1–44	64–80	—	92–217	—	224–355	378–432	502–544	965–1822	1973–2640	5420–5608
pBEn-SBP-SET2c	1–44	64–80	—	92–217	—	224–355	379–433	503–545	966–1823	1947–2641	4521–5609
pBEn-SBP-SET3a	1–44	64–80	—	—	92–217	224–355	377–431	501–543	964–1821	1972–2639	4519–5607
pBEn-SBP-SET3b	1–44	64–80	—	—	92–217	224–355	378–432	502–544	965–1822	1973–2640	4520–5608
pBEn-SBP-SET3c	1–44	64–80	—	—	92–217	224–355	379–433	503–545	966–1823	1974–2641	4521–5609
pBEn-SET1a	1–44	64–80	92–217	—	—	—	239–293	363–405	826–1683	1834–2501	4381–5469
pBEn-SET1b	1–44	64–80	92–217	—	—	—	240–294	364–406	827–1684	1835–2502	4382–5470

	T7 promoter with *lac* operon	extended ribosome binding site	solubility enhancement tag 1 (SET1 tag)	solubility enhancement tag 2 (SET2 tag)	solubility enhancement tag 3 (SET3 tag)	streptavidin binding peptide (SBP tag)	multiple cloning site	T7 terminator	ampicillin resistance (*bla*) ORF	pBR322 origin of replication	*lacI* repressor ORF
pBEn-SET1c	1–44	64–80	92–217	—	—	—	241–295	365–407	828–1685	1836–2503	4383–5471
pBEn-SET2a	1–44	64–80	—	92–217	—	—	239–293	363–405	826–1683	1834–2501	4381–5469
pBEn-SET2b	1–44	64–80	—	92–217	—	—	240–294	364–406	827–1684	1835–2502	4382–5470
pBEn-SET2c	1–44	64–80	—	92–217	—	—	241–295	365–407	828–1685	1836–2503	4383–5471
pBEn-SET3a	1–44	64–80	—	—	92–217	—	239–293	363–405	826–1683	1834–2501	4381–5469
pBEn-SET3b	1–44	64–80	—	—	92–217	—	240–294	364–406	827–1684	1835–2502	4382–5470
pBEn-SET3c	1–44	64–80	—	—	92–217	—	241–295	365–407	828–1685	1836–2503	4383–5471
pBEc-SBP	2–45	65–81	—	—	—	153–284	87–128	296–338	759–1616	1767–2434	4314–5402
pBEc-SBP-SET1	2–45	65–81	294–419	—	—	153–284	87–128	440–482	903–1760	1911–2578	4469–5546
pBEc-SBP-SET2	2–45	65–81	—	294–419	—	153–284	87–128	440–482	903–1760	1911–2578	4458–5546
pBEc-SBP-SET3	2–45	65–81	—	—	294–419	153–284	87–128	440–482	903–1760	1911–2578	4458–5546
pBEc-SET1	2–45	65–81	153–278	—	—	—	87–128	299–341	762–1619	1770–2437	4317–5405
pBEc-SET2	2–45	65–81	—	153–278	—	—	87–128	299–341	762–1619	1770–2437	4317–5405
pBEc-SET3	2–45	65–81	—	—	153–278	—	87–128	299–341	762–1619	1770–2437	4317–5405

BL21 EXPRESSION STRAINS

BL21 expression strains are recommended for use with the VariFlex vectors because of their compatibility with pET-derived vector features. The Agilent BL21-Gold(DE3) expression strain (Catalog #230132), derived from the *E. coli* B strain BL21, is recommended as a general protein expression strain. This strain is deficient in *lon* protease as well as the *ompT* outer membrane protease that can degrade proteins during purification.[6,8] The BL21(DE3) strains[4, 8] carry a lambda DE3 lysogen that has the phage 21 immunity region, the *lacI* gene, and the *lacUV5*-driven T7 RNA polymerase expression cassette. On induction with IPTG, the *lacUV5* promoter is derepressed, allowing overexpression of T7 RNA polymerase and expression of the T7-promoted target gene. The BL21-Gold-derived expression strains feature the Hte phenotype which increases the transformation efficiency of the cells. In addition, the gene that encodes endonuclease I (*endA*), which rapidly degrades vector DNA isolated by most miniprep procedures, is inactivated in the BL21-Gold(DE3) expression strain. Besides the general protein expression strains, there are a variety of BL21 host strains designed to address specific protein expression problems. These problems include the toxicity of the gene product and the availability of codons.

Many genes that are expressed from the very strong T7 promoter can be toxic to the *E. coli* host cells. When using the BL21-Gold(DE3) strain as the primary host strain for cloning, some caution should be exercised because even low-level expression can result in accumulation of a toxic gene product.

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

In cases in which target genes are too toxic to allow plasmids to be established in DE3 lysogens, T7 RNA polymerase can be delivered to the cell by infection with the bacteriophage CE6 by using the methods outlined in the *Lambda CE6 Induction Kit* Instruction Manual (Catalog #235200),

which is compatible with the VariFlex expression vectors. By using the method employed by the Lambda CE6 induction kit, no T7 RNA polymerase is present in the cell until the desired time of induction. The bacteriophage CE6 expresses T7 RNA polymerase from the lambda p_L and p_I promoters and carries the *Sam7* lysis mutations. This bacteriophage will allow effective expression of target genes in BL21 cells and presumably other nonrestricting hosts that absorb lambda. The phage can be propagated in the LE392 host strain [e14- (McrA-) *hsdR514 supE44 supF58 lacYI*],[9] which suppresses the *Sam7* mutation and therefore allows lysis of infected cells.

BL21 expression strains addressing codon usage issues are also available. Efficient production of heterologous proteins in *E. coli* is frequently limited by the rarity of certain tRNAs that are abundant in the organisms from which the heterologous proteins are derived. Forced high-level expression of heterologous proteins can deplete the pool of rare tRNAs and stall translation, resulting in low protein yields. Availability of tRNAs allows high-level expression of many heterologous recombinant genes in BL21-CodonPlus cells that are poorly expressed in conventional BL21 strains. BL21-CodonPlus strains are engineered to contain extra copies of genes that encode the tRNAs that most frequently limit translation of heterologous proteins in *E. coli*.

PREPARING THE VECTORS

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

- Any of the sites in the MCS can be used for the pBEn vectors; however, ensure that the proper coding sequence of the insert is in frame with the N-terminal tag (see the MCS regions in Figure 2).

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

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

LIGATING THE INSERT

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

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

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

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

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

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

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

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

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

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

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

TRANSFORMING THE LIGATION REACTIONS

Following subcloning into a routine cloning host strain, positive transformants are then used to transform a protein expression strain such as BL21-Gold(DE3) competent cells. A transformation protocol is provided for Agilent's BL21-Gold(DE3) competent cells (Catalog #230132). If transforming different competent cells, follow the manufacturer's recommendations.

Transformation Guidelines

Storage Conditions

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

Aliquoting Cells

When aliquoting, keep the competent cells on ice at all times. It is essential that the 14-ml BD Falcon polypropylene round-bottom tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at least 100 µl of competent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of 14-ml BD Falcon Polypropylene Tubes

The use of 14-ml BD Falcon polypropylene round-bottom tubes for transformation of Statagene's BL21-Gold(DE3) competent cells is imperative. The duration of the heat-pulse step is critical and is optimized for the thickness and shape (i.e., the round bottom) of these tubes.

Quantity of DNA Added

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

Length of the Heat Pulse

Optimal transformation efficiencies are observed when transformation reactions are heat-pulsed for 20–25 seconds at 42°C. Transformation efficiencies decrease sharply when the duration of the heat pulse is <20 seconds or >25 seconds.

Transformation Protocol

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

   Note *Store the competent cells **on ice at all times** while aliquoting. It is essential that the 14-ml BD Falcon polypropylene tubes are placed on ice before the competent cells are thawed and that 100 µl of competent cells are aliquoted directly into each **prechilled** polypropylene tube.*

2. Gently mix the competent cells. Aliquot 100 µl of the competent cells into the appropriate number of prechilled 14-ml BD Falcon polypropylene tubes.

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

4. Incubate the reactions on ice for 30 minutes.

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

6. Incubate the reactions on ice for 2 minutes.

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

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

   To plate the cells transformed with the pUC18 control vector, first place a 195-µl pool of SOC medium on an LB-ampicillin agar plate. Add 25 µl of the control transformation reaction to the pool of SOC medium. Use a sterile spreader to spread the mixture.

9. Incubate the plates overnight at 37°C. For the control pUC18 transformation reaction, ≥250 cfu are expected, indicating a transformation efficiency of $\geq 1 \times 10^8$.

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

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

INDUCTION OF THE TARGET PROTEIN USING IPTG

The following induction protocol is a general guide for expression of genes under the control of IPTG-inducible promoters on an analytical scale (1-ml of induced culture). Most commonly, this protocol is used to analyze protein expression of individual transformants of BL21(DE3) host strains in combination with vectors containing T7 promoter constructs (e.g., pBE vectors).

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

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

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

2. Following overnight incubation, pipet 50 µl of each culture into fresh 1-ml aliquots of NZY broth containing no selection antibiotics. Incubate these cultures with shaking at 220–250 rpm at 30°C for 3 hours.

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

4. To the rest of the culture in each tube add IPTG to a final concentration of 0.1–1 mM. Incubate with shaking at 220–250 rpm at 30°C for 1–3 hours.

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

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

AFFINITY PURIFICATION OF THE SBP-TAGGED PROTEIN

Preparing the Streptavidin Resin

Note *The volumes of resin and buffer given should be optimized to your experimental parameters.*

1. Centrifuge 50 µl of 50% streptavidin resin slurry (per 1 ml culture) at 1500 × *g* for 5 minutes to collect the resin. Discard the supernatant to remove the ethanol storage buffer. Resuspend the resin in 50–100 µl of streptavidin binding buffer (see *Preparation of Media and Reagents*). Repeat this wash step.

2. Collect the resin by centrifugation at 1500 × *g* for 5 minutes. Discard the supernatant and resuspend the resin in 25 µl of streptavidin binding buffer.

Purifying the SBP-tagged Protein Using Streptavidin Resin

1. Following induction by IPTG, pellet the cells by centrifugation at 2000 × *g* for 15 minutes. Discard the supernatant.

2. Resuspend the cells in 500 µl of streptavidin binding buffer and protease inhibitor(s).

   Note *If desired, lysozyme may be added to this mixture, and requires incubation on ice for 15 minutes.*

3. Lyse the cells by sonication.

4. Pellet the cell debris by centrifugation for 5 minutes at 12,000 × *g*. Retain the supernatant, which contains the expressed proteins.

5. For each 1 ml culture, add the supernatant to 50 µl of the prepared streptavidin resin (50% slurry). Rotate the tube at 4°C for 30 minutes to allow the tagged proteins to bind to the resin.

6. Collect the resin by centrifugation at 1500 × g for 5 minutes. Remove and freeze the supernatant for possible analysis later. Resuspend the resin in 1 ml of streptavidin binding buffer by rotating the tube at 4°C for 5 minutes.

7. Repeat step 6 twice, for a total of three washes.

8. Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant. Add 100 µl of streptavidin low salt elution buffer[§] to the resin. For cases when the protein fails to elute completely from the resin, use the high salt elution buffer[§].

9. Rotate the tube at 4°C for 30 minutes to elute the protein.

10. Collect the resin by centrifugation at 1500 × g for 5 minutes. Carefully transfer the supernatant to a fresh tube (the supernatant contains the eluted protein).

REMOVING THE TAGS WITH THROMBIN

Ideal digestion conditions will vary between proteins and should be optimized for each fusion protein. We recommend starting with a 1:500 thrombin-to-fusion protein ratio and analyzing the reaction products at various time points from several minutes to 24 hours following the addition of thrombin. A lower thrombin-to-target ratio (e.g., 1:50) may be used to decrease long reaction times. Dialyze or dilute the tagged fusion protein into thrombin cleavage buffer (see *Preparation of Media and Reagents*). Add the thrombin to the reaction tube and incubate at room temperature until cleavage is complete. Determine the efficiency of proteolytic removal of the tag(s) by SDS–PAGE analysis.

Note *Thrombin may be inactivated by the addition of protease inhibitor(s).*

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

TROUBLESHOOTING

Observations	Suggestions
Vector instability	Unstable DNA sequence. Prior to induction of cultures, assay for colony formation by plating cells on an LB plate and an LB-ampicillin plate. If the vector contains unstable DNA sequence one should observe colony formation on the LB plate, and reduced colony formation on the LB-ampicillin plate.
	Overexpression of toxic proteins. Prior to induction of cultures, assay for colony formation by plating cells on an LB-ampicillin plate and an LB-ampicillin plate containing IPTG. If the insert codes for a protein that is toxic to the cells, overexpression of the toxic protein should result in reduced colony formation on an LB-ampicillin plate containing IPTG as compared to cells plated on the LB-ampicillin plate.
	More tightly controlled induction may be achieved by performing induction by infecting BL21 cells with the bacteriophage CE6.
Problems associated with induction time	Depends on the physicochemical characteristics of the protein and toxicity of the protein to *E. coli*. In certain cases, accumulation of target protein may kill cells at saturation while allowing normal growth in logarithmically growing cultures, while in other cases target protein may continue to accumulate in cells well beyond the recommended 3-hour induction period. To determine the optimal induction period, a time course may be carried out during which a small portion of the culture is analyzed by SDS–PAGE at various times following induction.
Inclusion bodies	Improper folding in *E. coli* and/or bacterial aggregation due to the physical properties of the protein. In some cases, protein may form insoluble inclusion bodies at 37°C. In many cases, this protein may be soluble and active if the induction is carried out at 30°C.
	Use the expression vectors containing the SET tags, which may reduce insoluble inclusion body formation.
Precipitation of fusion protein observed in elution fractions	Insufficient ionic strength in the elution buffer and/or the pH of the elution buffer is inappropriate for the pH of the fusion protein. Optimize the buffer system to correct the ionic strength in the elution buffer or correct the pH of the elution buffer affecting the pH of the fusion protein.
Contaminating proteins coeluting with fusion protein	The use of nonionic detergents, such as NP-40 and Triton X-100, at 0.1% may be effective in the elimination of contaminating proteins.
Protein fails to elute completely from the resin	Protein precipitated on the resin. Increase NaCl concentration of the elution buffer. Add detergent to the elution buffer.
	Biotin concentration in the elution buffer too low. Ensure that biotin concentration in streptavidin elution buffer is 2 mM.
Incomplete proteolytic cleavage	The efficiency of proteolytic removal of the tags by thrombin will vary from protein to protein, and in some cases, the conformation of the protein may inhibit accessibility of the thrombin cleavage target site for the enzyme. Longer incubation times or higher concentrations of protease may help.
	Positioning of the tag at the opposite terminus of the protein of interest by recloning the insert into the appropriate protein expression vector may increase accessibility of the target site.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Cool to 55°C.
Add appropriate antibiotic.
Pour into petri dishes (~25 ml/100-mm plate)

NZY Broth (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave

NZY –Ampicillin Broth (per Liter)

1 liter of NZY broth, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin

LB–Ampicillin Agar (per Liter)

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

NZY –Carbenicillin Broth (per Liter)

1 liter of NZY broth, autoclaved
Autoclave
Cool to 55°C
Add 10 ml of 10-mg/ml-filter-sterilized carbenicillin

SOB Medium (per Liter)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Autoclave
Add 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$ prior to use
Filter sterilize

SOC Medium (per 100 ml)

SOB medium
Add 1 ml of a 2 M filter-sterilized glucose solution or 2 ml of 20% (w/v) glucose prior to use
Filter sterilize

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

Streptavidin Binding Buffer

10 mM Tris HCl, pH 8.0
150 mM NaCl

Low-Salt Streptavidin Elution Buffer

10 mM Tris HCl, pH 8.0
150 mM NaCl
2 mM biotin

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction*

High-Salt Streptavidin Elution Buffer

10 mM Tris HCl, pH 8.0
1 M NaCl
2 mM biotin

Thrombin Cleavage Buffer

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

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ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.