invitrogen™

pBAD/Thio-E Echo™-Adapted Expression Vector

For expression of the gene of interest in E. coli using the Echo™ Cloning System

Catalog nos. ET100-XX

Version H
29 December 2010
25-0329

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

Several pBAD/Thio-E Echo™ Cloning System Kits are available. The table below lists the kits that include the pBAD/Thio-E Echo™-Adapted Expression Vector.

Kit	Reagents Supplied	Catalog nos.
pBAD/Thio-E Echo™-Adapted Expression Vector Kit	pBAD/Thio-E vector Expression Control vector Cre Recombinase and 10X buffer Trx Forward Sequencing Primer 20% L-Arabinose	ET100-01
pBAD/Thio-E Echo™-Adapted Expression Vector Kit with a choice of Donor Vector Kit and One Shot® TOP10 Chemically Competent *E. coli* (see page viii for more information on donor vectors)	pUni/V5-His TOPO® TA Cloning Kit	ET100-10C
	pUniBlunt/V5-His TOPO® Cloning Kit	ET100-20C
	pUni/V5-His A, B, and C	ET100-30C
	pUniD/V5-His TOPO® Cloning Kit	ET100-40C

Shipping and Storage

The pBAD/Thio-E Cloning and Expression Kit is shipped on dry ice. Upon receipt, store the pBAD/Thio-E reagents at -20°C. Store the One Shot® Competent *E. coli* at -80°C.

pBAD/Thio-E Reagents

pBAD/Thio-E reagents are listed below. Store at -20°C.

Item	Concentration	Amount
pBAD/Thio-E	Supercoiled, lyophilized in TE, pH 8	20 µg
Cre Recombinase	Please check the label for exact concentration of the enzyme Enzyme supplied in: 50 mM Tris-HCl, pH 8.0 5 mM EDTA 1 mM EGTA 10 mM β-mercaptoethanol 20% Glycerol	15 µl
10X Recombinase Buffer	500 mM Tris-HCl, pH 7.5 100 mM $MgCl_2$ 300 mM NaCl 1.0 mg/ml BSA	25 µl

continued on next page

Kit Contents and Storage, continued

pBAD/Thio-E Reagents, continued

Item	Concentration	Amount
Trx Forward Sequencing Primer (18 mer)	Lyophilized in TE Buffer, pH 8 (5´-TTCCTCGACGCTAACCTG-3´)	2 µg (371 pmoles)
Expression control (pBAD/Thio-E/Uni-CAT)	Supercoiled, lyophilized in TE, pH 8	20 µg
20% L-Arabinose	20% in sterile water	1 ml

One Shot® Reagents (Optional)

The table below describes the items included in the One Shot® Competent *E. coli* kit.

Store at -80°C.

Item	Composition	Amount
SOC Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 *E. coli*	--	11 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

TOP10: Use this strain for general cloning of your gene of interest. Note: This strain should not be used for the growth and transformation of donor vectors.

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Qualifying the Product

Vectors

pBAD/Thio-E and pBAD/Thio-E/Uni-CAT are qualified by restriction digest. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pBAD/Thio-E	pBAD/Thio-E/Uni-CAT
Afl II	3296 bp, 1099 bp	Not tested
*Hin*d III (linearizes)	4395 bp	4690 bp, 2674 bp
Pme I (linearizes)	4395 bp	Not tested
*Eco*R V	Not tested	4818 bp, 2546 bp
Xho I (linearizes)	Not tested	7364 bp

Primers

The Trx Forward Sequencing primer has been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

Cre Recombinase

Purity: >95% homogeneity

Endonuclease activity: Negative

Exonuclease activity: Negative

Functional Assay: Cre recombinase is qualified using the assay on page 5 of this manual. The donor vector is pUni/*lacZ* and the acceptor vector is pcDNA3.1-E™. Five microliters of the recombination reaction is transformed into 50 µl TOP10 One Shot® competent *E. coli* using the protocol on page 7. Twenty-five µl of the transformation reaction is plated on LB plates containing 50 µg/ml kanamycin (performed in duplicate). One microliter of Cre recombinase should yield >500 blue, kanamycin-resistant transformants.

One Shot® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

Accessory Products

Products Available Separately

The table below lists additional products that may be useful for characterizing, detecting, or purifying your protein.

Product	Amount	Catalog No.
PIR1 One Shot® *E. coli* (chemically competent)	11 x 50 µl	C1010-10
Cre Recombinase	10 reactions	R100-10
Anti-His(C-term) Antibody	50 µl*	R930-25
Anti-His(C-term)-HRP Antibody	50 µl*	R931-25
Anti-Thio™ Antibody	50 µl*	R920-25
Anti-V5 Antibody	50 µl*	R960-25
Anti-V5-HRP Antibody	50 µl*	R961-25
ProBond™ Purification System (includes precharged ProBond™ resin, 6 columns, and buffers for purification under native and denaturing conditions)	6 purifications	K850-01
ProBond™ Resin	50 ml 150 ml	R801-01 R801-15
Purification Columns	50	R640-50

*Quantity supplied is sufficient for 25 western blots.

Donor Vectors

The table below lists a variety of donor vectors currently available from Invitrogen to facilitate cloning of your gene of interest for use with Echo™ Cloning System.

Product	Application	Quantity	Catalog no.
pUni/V5-His-TOPO® TA Cloning Kit	Cloning A-tailed PCR products	10 reactions	ET001-10
pUniBlunt/V5-His-TOPO® Cloning Kit	Cloning blunt PCR products	10 reactions	ET002-10
pUniD/V5-His-TOPO® Cloning Kit	Directional cloning of blunt PCR products	10 reactions	ET004-10
pUni/V5-His A, B, and C	Cloning DNA fragments using restriction enzymes	10 reactions	ET003-10

Purchaser Notification

Limited Use Label License No: 22 Vectors and Clones Encoding Histidine Hexamer	This product is licensed under U.S. and foreign patents Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.
Limited Use Label License No: 29 Thiofusion ™ Expression System	The ThioFusion™ Expression System is licensed under U.S. patent from Genetics Institute, Inc. for research use only. Licenses for commercial manufacture or use may be obtained directly from Genetics Institute, Inc., 87 Cambridgepark Drive, Cambridge, MA 02140.
Limited Use Label License No: 119 Echo™ Cloning Products	No license is conveyed to use this product with any recombination sites other than those purchased from Invitrogen Corporation or its authorized distributor. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose.

Introduction

Overview

Introduction

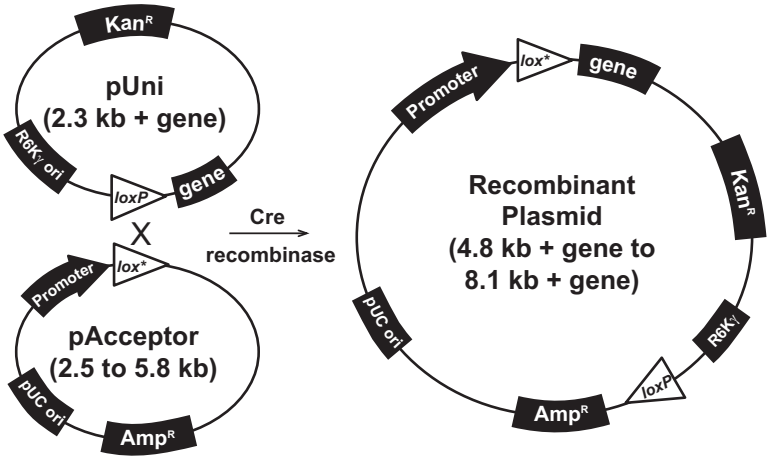
The Echo™ Cloning System allows direct recombination of your gene of interest downstream of an appropriate promoter for expression in the host system of choice. pBAD/Thio-E is a member of the Echo™ Cloning System family of expression vectors, specifically designed for tightly regulated expression in *E. coli*. Expression in *E. coli* is driven by the *ara*BAD promoter (P_{BAD}). The AraC gene product encoded on the pBAD/Thio-E plasmid positively regulates this promoter. Recombinant proteins are expressed as fusions to His-Patch thioredoxin for high-level expression.

The Echo™ Cloning System

The Echo™ Cloning System is based on the univector plasmid-fusion system (UPS) described by Elledge and coworkers to quickly and easily recombine a gene of interest into a series of recipient (acceptor) vectors (Liu *et al.*, 1998; Liu *et al.*, 1999). The system consists of the univector (donor) vector containing the gene of interest and recipient (acceptor) vectors containing various regulatory sequences for expression in the host of choice. The system utilizes the *cre-lox* site-specific recombination system of bacteriophage P1 to recombine the gene of interest into the acceptor vector of choice (Abremski *et al.*, 1983; Sternberg *et al.*, 1981a). The product of the *cre* gene is a site-specific recombinase that catalyzes recombination between two 34 bp *loxP* or *loxH* sequences to resolve P1 dimers generated by replication of circular lysogens.

Plasmid Fusion

The donor vector (pUni) and the acceptor vector (i.e., pBAD/Thio-E) each contain a *lox* site. The donor vector contains a *loxP* site, while the acceptor vector contains a *loxP* or a *loxH*. You may construct the donor vector containing the gene of interest via the TOPO® Cloning method or traditional restriction enzyme-mediated cloning. pBAD/Thio-E contains the appropriate transcription regulatory sequences that will control expression of the gene of interest in *E. coli*. The vector also carries translation initiation and additional coding sequences for generating fusion proteins. A unique *loxP* site is located downstream of the regulatory sequences. By mixing the donor vector containing the gene of interest with pBAD/Thio-E in the presence of Cre recombinase, a plasmid fusion is created that expresses the gene of interest in *E. coli*. A generic diagram is shown below.



*lox** = *loxP* or *loxH* depending on acceptor vector

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Overview, continued

loxP

The sequence of the *loxP* site is shown below. It consists of a 34 bp sequence containing a 13 bp inverted repeat separated by an 8-bp spacer region (Hoess *et al.*, 1982). The inverted repeat (underlined) may form a stem and loop structure that may reduce expression of the gene of interest in some cases. Expression from pBAD/Thio-E was compared to expression from the parent plasmid (pBAD/Thio), and found to be essentially equivalent.

loxP: ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA T

Cre Recombinase

Cre recombinase (MW = 35 kDa) is a site-specific recombinase that binds to specific sequences (*loxP* and *loxH* sites), brings together the target sites, cleaves, and covalently attaches to the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel (recombinant) form. A nucleophilic hydroxylated tyrosine initiates the DNA cleavage event by attack on a specific phosphodiester bond followed by the covalent attachment of the recombinase to the target sequence through a phosphoamino acid bond (Abremski and Hoess, 1992; Argos *et al.*, 1986). The reaction does not require any host factors or ATP, but does require Mg^{2+} or spermidine for activity (Abremski *et al.*, 1983). Recombination between two supercoiled substrates, each containing a *loxP* or *loxH* site, results in a supercoiled dimer. The extent of the reaction is 10-20% and appears to be stoichiometric (Abremski and Hoess, 1984; Abremski *et al.*, 1983).

Selection of Recombinants

By fusing the two plasmids, kanamycin resistance is now linked to the pUC origin of replication. The recombination reaction is transformed into TOP10 *E. coli* and recombinants selected by plating the transformation reaction onto plates containing kanamycin. Because the donor plasmid carries the R6Kγ origin of replication, it will not propagate in *E. coli* strains such as TOP10 which do not carry the *pir* gene. In addition, the acceptor vector, which carries the ampicillin resistance gene will not be selected. Therefore every colony that is selected on kanamycin will represent a recombined fusion plasmid.

pBAD/Thio-E

pBAD/Thio-E contains the features listed below.

- *araBAD* promoter for regulated, high-level expression in *E. coli*
- An N-terminal peptide encoding His-Patch thioredoxin for efficient translation and, in some cases, increased solubility
- Enterokinase cleavage site for release of the N-terminal peptide
- A *loxP* site for plasmid fusion
- The pUC origin for high copy replication and maintenance in most *E. coli* strains
- The *bla* gene for ampicillin resistance

Other Echo™-adapted acceptor vectors are available separately and are provided with their own manuals. For more information on other available acceptor vectors, please visit our Web site (www.invitrogen.com) or call Technical Service (see page 22).

continued on next page

Overview, continued

Regulation of Expression by Arabinose

In the presence of arabinose, expression from P_{BAD} is induced while only very low levels of transcription are observed from P_{BAD} in the absence of arabinose (Lee, 1980; Lee *et al.*, 1987). Uninduced levels are repressed even further by growth in the presence of glucose. Glucose reduces the levels of 3´, 5´-cyclic AMP, lowering expression from the catabolite-repressed P_{BAD} promoter (Miyada *et al.*, 1984). By varying the concentration of arabinose, protein expression levels can be optimized to ensure maximum expression of protein. In addition, the tight regulation of P_{BAD} by AraC is useful for expression of potentially toxic or essential genes (Carson *et al.*, 1991; Dalbey and Wickner, 1985; Guzman *et al.*, 1992; Kuhn and Wickner, 1985; Russell *et al.*, 1989; San Millan *et al.*, 1989). For more information on the mechanism of expression and repression of the *ara* regulon, please see page 21 or refer to Schleif, 1992.

Thioredoxin

The 11.7 kDa thioredoxin protein is found in yeast, plants, and mammals, as well as in bacteria. It was originally isolated from *E. coli* as a hydrogen donor for ribonuclease reductase (for a review, please see Holmgren, 1985). The gene has been completely sequenced (Wallace and Kushner, 1984). The protein has been crystallized and its three-dimensional structure determined (Katti *et al.*, 1990). When overexpressed in *E. coli*, thioredoxin is able to accumulate to approximately 40% of the total cellular protein and still remains soluble. Thioredoxin is used to increase translation efficiency, and in some cases, solubility, of eukaryotic proteins expressed in *E. coli*. Murine interleukin-2, human interleukin-3, murine interleukin-4, murine interleukin-5, human macrophage-colony stimulating factor, murine steel factor, murine leukemia inhibitory factor and human bone morphogenetic protein-2 are some of the proteins that have been produced as soluble C-terminal fusions to the thioredoxin protein in *E. coli* (LaVallie *et al.*, 1993).

His-Patch Thioredoxin

To create a metal binding domain in the thioredoxin protein, the glutamate residue at position 32 and the glutamine residue at position 64 were mutated to create histidine residues. When His-Patch thioredoxin folds, the histidines at positions 32 and 64 interact with a native histidine at position 8 to form a "patch". This histidine patch has high affinity for divalent cations (Lu *et al.*, 1996). His-Patch thioredoxin (HP-thioredoxin) proteins can therefore be purified on metal-chelating resins (e.g., ProBond™) under native conditions (see ordering information, page viii).

continued on next page

Overview, continued

Experimental Outline

The table below describes the general steps needed to recombine, transform, and express your protein of interest.

Important

Please note that under denaturing conditions, the Ni^{2+} binding site encoded by the histidine 'patch' will be destroyed because the HP-thioredoxin protein will be denatured. The binding of nickel ion to the 6xHis tag is not affected by denaturing conditions.

If you want to purify your protein under denaturing conditions using metal-chelating resins, be sure to fuse your gene of interest to the C-terminal peptide from the donor vector. The C-terminal peptide encodes a 6xHis tag that can be use to purify the protein of interest under denaturing conditions.

Methods

Recombining Your Gene into pBAD/Thio-E

Introduction

At this point you should have a plasmid preparation of your donor vector in addition to pBAD/Thio-E. Please review the information below and on the next page before performing the recombination reaction.

Preparing pBAD/Thio-E

To prepare pBAD/Thio-E for use, add 20 µl of sterile, deionized water to the lyophilized plasmid. This will yield a 1 µg/µl stock solution. You can further dilute a small aliquot or use as is. Store at -20°C when you are finished.

If you wish to propagate this plasmid or prepare more plasmid DNA, you may transform this plasmid into TOP10 *E. coli* as described on pages 6-7. Use 10-100 ng plasmid for transformation and select on LB plates containing 50-100 µg/ml ampicillin.

Before Staring

You will need the following reagents and equipment.

- 100 ng of your donor vector
- 100 ng of pBAD/Thio-E (included in kit)
- Microcentrifuge tubes
- Heat blocks set at 37°C and 65°C
- Ice bucket with ice
- Cre recombinase (included in the kit)
- 10X Recombinase buffer (included in the kit)

Recombination Reaction

1. Set up each 20 µl recombination reaction **on ice** as follows.

Donor vector (100 ng)	x µl
pBAD/Thio-E (100 ng)	y µl
10X Recombinase buffer	2 µl
Deionized wateradd to a total volume of	17 µl
Cre Recombinase	1 µl
Final Volume	20 µl

2. Incubate at 37°C for 20 minutes.
3. Incubate at 65°C for 5 minutes to inactivate the recombinase.
4. Place tube on ice and proceed to **Transformation**, next page. If you run out of time, you may store the recombination reaction at +4°C or -20°C overnight. Longer storage times have not been tested.

Transforming the Recombination Reaction

Introduction

Once you have performed the recombination reaction you are ready to transform your *E. coli* host. We recommend TOP10 *E. coli* (available with the kit) for transformation and induction with arabinose but other strains may be used. Strains should be *endA* and *recA* to ensure quality plasmid preparations and reduce the chances of recombination, respectively. In addition, we also recommend that you use a strain that is deficient in arabinose metabolism (*araBAD*), but can still take up arabinose (*araEFGH*$^{+}$). Check the genotype of your strain before preparing competent cells.

Materials Supplied by the User

In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath
- LB plates containing 50 μg/ml kanamycin (see **Important**, below)
- 37°C shaking and non-shaking incubator
- SOC (supplied in the One Shot® kit)

Important

It is important to select the fusion plasmid using kanamycin. Remember that the donor vector contains the R6Kγ origin. This origin can only be maintained in *E. coli* strains containing the *pir* gene. After the donor vector and pBAD/Thio-E have recombined to form the fusion plasmid, the kanamycin resistance gene (from the donor vector) is linked to the pUC origin (from pBAD/Thio-E). The fusion plasmid can be maintained in *E. coli* strains that do not contain the *pir* gene (i.e. TOP10). By selecting for kanamycin resistance, you ensure that only colonies containing the fusion plasmid are selected..

Preparing for Transformation

The following transformation protocol is for use with the TOP10 One Shot® competent cells available with the kit. If you are using other competent cells, please follow the manufacturer's protocol.

For each transformation, you will need one vial of competent cells and two selective plates.

1. Equilibrate a water bath to 42°C.
2. Thaw the vial of SOC medium from the One Shot® box and bring to room temperature.
3. Warm LB plates containing 50 μg/ml kanamycin at 37°C for 30 minutes.
4. Thaw **on ice** 1 vial of One Shot® TOP10 *E. coli* for each transformation.

continued on next page

Transforming the Recombination Reaction, continued

One Shot® Transformation Reaction

1. Add 5 µl of the recombination reaction to a vial of One Shot® TOP10 *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Heat-shock the cells for 30 seconds at 42°C without shaking.
3. Immediately transfer the tubes to ice.
4. Add 450 µl of room temperature SOC medium.
5. Cap the tube tightly and shake the tube horizontally at 37°C for 45 minutes.
6. Spread 50 µl from each transformation on a prewarmed plate. Pellet the remaining cells, resuspend the cell pellet in 50 µl SOC and plate. Incubate overnight at 37°C.
7. An efficient recombination reaction will produce hundreds of colonies. Pick ~5 colonies for analysis.

Analyzing Positive Clones

1. Culture 5 colonies overnight in 2-5 ml LB or SOB medium containing 50 µg/ml kanamycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis. Use an enzyme or enzymes that cut once in the donor vector and once in the acceptor vector to yield two fragments that are distinguishable from one another. Please note that other strategies are possible.
4. (Optional) To sequence the fusion plasmid to confirm the fusion junctions, use the Trx Forward and Uni1 Forward sequencing primers. Please refer to the diagram on the next page for the sequence around the pBAD/Thio-E *loxP* site. Refer to the donor vector manual for the sequence around the donor vector *loxP* site.

If you need help with setting up restriction enzyme digests or DNA sequencing, please refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989)

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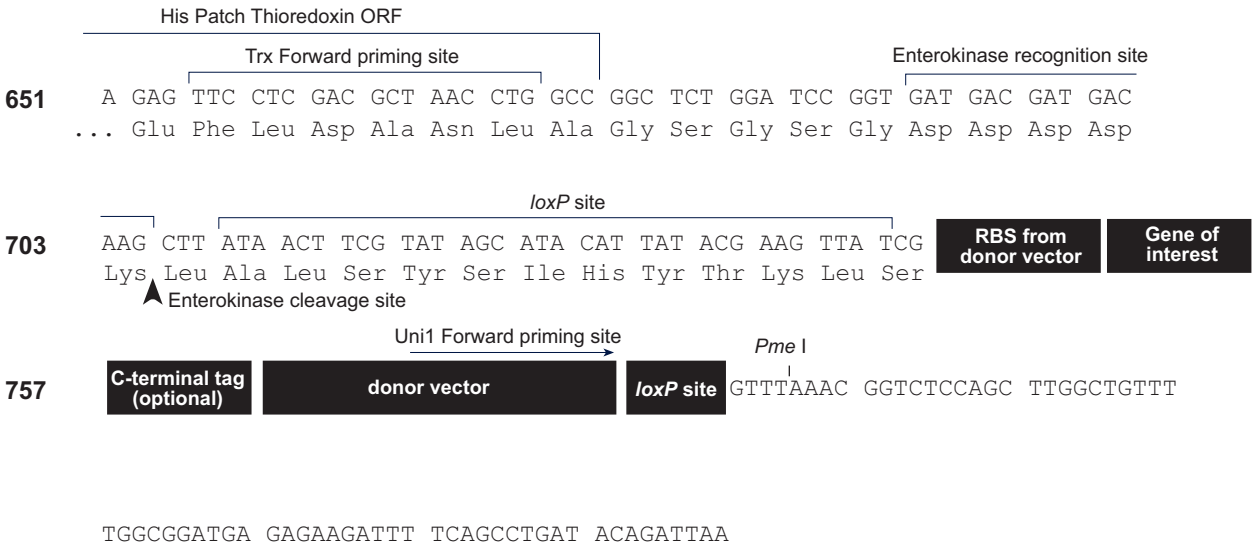
Transforming the Recombination Reaction, continued

Sequencing Your Construct

The sequence surrounding your insert is shown below. Unique restriction sites are labeled for your convenience. Please note that the complete sequence of pBAD/Thio-E can be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (page 22).



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Transforming the Recombination Reaction, continued

Fusion Vector Analysis

It should be clear from restriction analysis that you have a dimer plasmid consisting of the donor vector and pBAD/Thio-E. Occasionally, trimers will result. Trimers usually consist of two donor vector molecules and one acceptor molecule. In theory, trimers may result from two sequential fusion events or a single fusion event between a pre-existing monomeric substrate and a dimeric substrate. The production of trimers can be eliminated if gel-purified monomeric supercoiled DNA is used in the recombination reaction. Please note that trimers usually express as well as the dimer product.

Preparing a Glycerol Stock for Long-Term Storage

Once you have identified the correct clone, prepare a glycerol stock for long term storage.

1. Streak out the original colony on LB plates containing 50 μg/ml kanamycin to isolate single colonies.
2. Select a single colony and inoculate into 1-2 ml of LB containing 50 μg/ml kanamycin.
3. Grow overnight until culture is saturated.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C. (You may also want to store a stock of plasmid DNA at -20°C.)

Expressing Your Gene

Introduction

Since each recombinant protein has different characteristics that may affect optimal expression, it is helpful to vary the arabinose concentration and/or run a time course of expression to determine the best conditions for optimal expression of your particular protein. pBAD/Thio-E/Uni-CAT is included for use as a positive expression control. TOP10 cells may be used as a general host for expression.

pBAD/Thio-E/ Uni-CAT Vector

The positive control vector, pBAD/Thio-E/Uni-CAT, is included in the kit as an expression control. Details of this vector are provided on pages 18-19. Transform the vector (10 ng) into TOP10 cells using the procedure on page 7.

Basic Strategy

Once you have some clones that you wish to characterize, we recommend the following strategy to determine the optimal expression level.

1. **Pilot Expression.** In this expression experiment you will vary the amount of arabinose over a 10,000-fold range (0.00002% to 0.2%) to determine the approximate amount of arabinose needed for maximum expression of your protein. See next page for protocol.
2. To optimize expression of your protein, you may wish to try arabinose concentrations spanning the amount determined in Step 1. Or you may perform a time course.

Note: If your protein is insoluble, remember to analyze the supernatant and the pellet of lysed cells for expression of soluble protein (page 13).

Note

Expression of your protein with the N-terminal HP-thioredoxin peptide and the C-terminal tag (if included from the donor vector) will increase the size of your protein by 13 kDa and 3 kDa, respectively. Be sure to account for any additional amino acids between the tags and your protein.

continued on next page

Expressing Your Gene, continued

Before Starting

Be sure to have the following solutions and equipment on hand before starting the experiment:

- SOB or LB containing 50 μg/ml kanamycin (see **Recipes**, page 16)
- 37°C shaking incubator
- 20% L-arabinose (provided). Additional L-arabinose is available from Sigma (Catalog no. A3256).
- 37°C heat block or water bath
- 42°C water bath
- Liquid nitrogen
- 1X and 2X SDS-PAGE sample buffer
- Reagents and apparatus for SDS-PAGE gel
- Boiling water bath
- Lysis Buffer (see page 17 for recipe)
- Sterile water

Pilot Expression

In addition to testing your transformants, we recommend that you include the pBAD/Thio-E/ Uni-CAT vector as a positive control and cells without vector as a negative control.

1. For each transformant or control, inoculate 2 ml of SOB or LB containing 50 μg/ml kanamycin with a single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking (225-250 rpm) to OD_{600} = 1-2.
3. The next day, label five tubes 1 through 5 and add 10 ml of SOB or LB containing 50 μg/ml kanamycin.
4. Inoculate each tube with 0.1 ml of the overnight culture.
5. Grow the cultures at 37°C with vigorous shaking to an OD_{600} = ~0.5 (the cells should be in mid-log phase).
6. While the cells are growing, prepare four 10-fold serial dilutions of 20% arabinose with sterile water using aseptic technique (e.g. 2%, 0.2%, 0.02%, and 0.002%).
7. Remove a 1 ml aliquot of cells from each tube, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
8. Freeze the cell pellet at -20°C. This is the zero time point sample.

continued on next page

Expressing Your Gene, continued

Pilot Expression, continued

9. Use the stock solutions prepared in Step 6, previous page, and add arabinose to the five 9 ml cultures as follows. **Note**: For the positive and negative controls, it is not necessary to test all concentrations of arabinose. Use only the highest concentration of arabinose.

Tube	Stock Solution	Volume (ml)	Final Concentration
1	0.002%	0.09	0.00002%
2	0.02%	0.09	0.0002%
3	0.2%	0.09	0.002%
4	2%	0.09	0.02%
5	20%	0.09	0.2%

10. Grow at 37°C with shaking for 4 hours.
11. Take 1 ml samples at 4 hours and treat as in Step 7 and 8, previous page. You will have a total of 10 samples for each transformant and two samples for each control.

Preparing Samples

Prepare SDS-PAGE gels to analyze all the samples you collected. **Note**: If you wish to analyze your samples for soluble protein, please see page 13 for a protocol.

1. When all the samples have been collected from Steps 8 and 11, above, resuspend each pellet in 100 µl of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5-10 µl of each sample on an SDS-PAGE gel and electrophorese. Save your samples by storing at -20°C.

Analyzing Samples

1. Stain the gel with Coomassie® blue and look for a band of increasing intensity in the expected size range for the recombinant protein.
2. Use the positive control (pBAD/Thio-E/CAT) to confirm that growth and induction was done properly. The positive control should yield a 42 kDa protein when induced with 0.02% arabinose.
3. You should be able to determine the approximate arabinose concentration for maximum expression.

Low Expression

If you don't see any expression on a Coomassie®-stained gel, re-run your samples on an SDS-PAGE gel and perform a Western blot. Use antibody to your protein or any of the antibodies listed on page viii. **Note**: Remember that if your PCR product is in frame with the C-terminal peptide in the donor vector, you may use antibodies to either the epitope or to the 6xHis tag to detect your protein of interest.

If you still don't see expression of your protein, sequence your construct and make sure it is in frame with the N-terminal and/or C-terminal peptide.

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Expressing Your Gene, continued

Optimizing Expression

Once you have detected expression of your protein of interest, you may wish to perform some experiments to further optimize expression. Use the Pilot Expression protocol, but vary the arabinose concentration over a smaller range. For example, if you obtained the best expression at 0.002%, try 0.0004%, 0.0008%, 0.001%, 0.004%, and 0.008%.

Also you may perform a time course of induction over a 5 to 6 hour time period, taking time points every hour, to determine if varying the time increases expression.

If your protein is insoluble, you may wish to analyze the supernatant and pellet of lysed cells when you vary the arabinose concentration. Please refer to the protocol below to prepare your samples.

Remember to store your cell lysates at -20°C.

Preparing Samples for Soluble/Insoluble Protein

After collecting all of your samples from a time course experiment, prepare SDS-PAGE gels for analysis.

1. When all the samples have been collected, thaw and resuspend each pellet in 500 µl of Lysis Buffer (see **Recipes**, page 17).
2. Place sample on ice and sonicate solution 10 seconds.
3. Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at +4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Mix together equivalent amounts of supernatant and 2X SDS sample buffer and boil for 5 minutes.
5. Add 500 µl of 1X SDS-PAGE sample buffer to pellets from Step 3 and boil 5 minutes.
6. Load 10 µl of the supernatant sample and 5 µl of the pellet sample onto an SDS-PAGE and electrophorese.

Expressing Toxic Proteins

To ensure basal levels of expression, you may find it useful to utilize glucose to repress the *araBAD* promoter further. Follow the steps below to express your protein.

- Follow the Pilot Expression on page 11, substituting RM medium containing glucose (see page 17) to grow the cells.
- Be sure to monitor the OD_{600} as the cells will grow more slowly in RM medium.
- Induce with various concentrations of arabinose as described in the Pilot Expression.
- Monitor OD_{600} over time to be sure cells are growing.

Purifying Your Protein

Introduction

Once you have expressed your recombinant fusion protein, you are ready to purify your fusion protein using a metal-chelating resin such as ProBond™.

ProBond™

ProBond™ is a nickel-charged Sepharose® resin that can be used for affinity purification of fusion proteins containing the HP-thioredoxin leader peptide and/or a 6xHis tag. Proteins bound to the resin may be eluted with either low pH buffer or competition with imidazole or histidine. Please note that the capacity of ProBond™ is about 1 mg of protein per milliliter.

- To scale up your pilot expression for purification, see below.
- To order ProBond™ resin, see page viii.
- To purify your fusion protein using ProBond™, please refer to the ProBond™ Purification manual.
- To purify your fusion protein using another metal-chelating resin, please refer to the manufacturer's instructions.

Important

Please note that denaturing conditions will destroy the Ni^{2+} binding site created by the histidine 'patch' in HP-thioredoxin.

Additional Purification Steps

There may be cases when your specific HP-thioredoxin fusion protein may not be completely purified by metal affinity chromatography. Other protein purification techniques may be utilized in conjunction with ProBond™ to purify the fusion protein (Deutscher, 1990).

Scale-up of Expression for Purification on ProBond™

Depending on the expression level of your recombinant fusion protein, you may need to adjust the culture volume to bind the maximum amount of recombinant fusion protein to your column. For a 2 ml ProBond™ column, start with 50 ml of bacterial culture.

If you need to purify larger amounts of recombinant protein, you may need more ProBond™ resin. See page viii for ordering information.

To grow and induce a 50 ml bacterial culture:

1. Inoculate 2 ml of SOB or LB containing 50 μg/ml kanamycin with a single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking (225-250 rpm) to OD_{600} = 1-2.
3. The next day, inoculate 50 ml of SOB or LB containing 50 μg/ml kanamycin with 1 ml of the overnight culture.
4. Grow the culture at 37°C with vigorous shaking to an OD_{600} = ~0.5 (the cells should be in mid-log phase).
5. Add the optimal amount of arabinose to induce expression.
6. Grow at 37°C with shaking until the optimal time point is reached. Harvest the cells by centrifugation (3000 x g for 10 minutes at +4°C).
7. At this point, you may proceed directly to purification (ProBond™ Purification System manual) or store the cells at -80°C for future use.

continued on next page

Purifying Your Protein, continued

Removing the N-terminal Leader

The enterokinase recognition site in the HP-thioredoxin leader may be utilized to remove the leader sequence from your protein after purification. Please note that after digestion with enterokinase, there will be additional amino acids remaining at the N-terminus of the protein (see page 8).

To digest your fusion protein with enterokinase, please follow the manufacturer's recommendations.

A recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) is available from Invitrogen (Catalog no. E180-01). Instructions for digestion are included with the product. For more information, please contact Technical Service (page 22). To remove EKMax™ from the digest, EK-Away™ (Catalog no. R180-01) is also available.

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/ml of kanamycin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

SOB Medium (with Kanamycin)

SOB (per liter)

2% Tryptone
0.5% Yeast Extract
0.05% NaCl
2.5 mM KCl
10 mM $MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.5 with 5 M NaOH and add deionized water to 1 liter.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$. You may also add kanamycin to 50 µg/ml.
5. Store at +4°C. Medium is stable for only ~1 month.

continued on next page

Recipes, continued

RM Medium + Glucose

1X M9 Salts (see below for recipe for 10X M9 Salts)
2% Casamino Acids
0.2% glucose
1 mM $MgCl_2$
50 μg/ml kanamycin

1. For 1 liter of RM medium, mix 20 g Casamino Acids and 890 ml deionized water.
2. Autoclave 20 minutes on liquid cycle.
3. After the autoclaved solution has cooled, add the following sterile solutions aseptically:

10X M9 Salts	100 ml
1 M $MgCl_2$	1 ml
20% glucose	10 ml
50 mg/ml kanamycin	1 ml

4. Mix well and store medium containing kanamycin at +4°C. Medium is good for 1 month at +4°C.

10X M9 Salts

For 1 liter:

Na_2HPO_4	60 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g
Water	900 ml

1. Dissolve reagents in the water and adjust the pH to 7.4 with 10 M NaOH.
2. Add water to 1 liter and autoclave for 20 minutes on liquid cycle.
3. Store at room temperature.

Lysis Buffer

50 mM potassium phosphate, pH 7.8
400 mM NaCl
100 mM KCl
10% glycerol
0.5% Triton X-100
10 mM imidazole

1. Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:

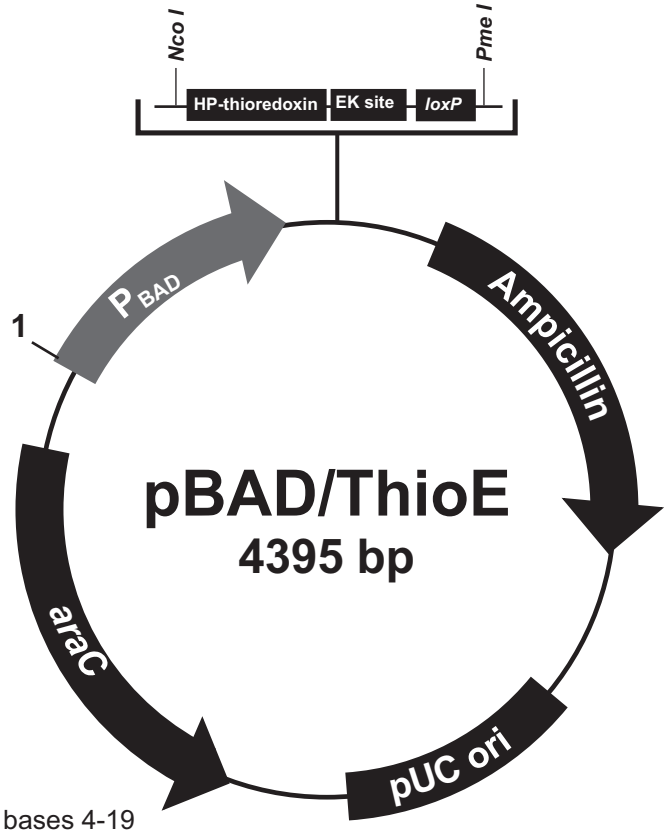
   0.3 ml KH_2PO_4
   4.7 ml K_2HPO_4
   2.3 g NaCl
   0.75 g KCl
   10 ml glycerol
   0.5 ml Triton X-100
   68 mg imidazole

3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
4. Store at +4°C.

Map and Features of pBAD/Thio-E

pBAD/Thio-E Map

The map below shows the features of pBAD/Thio-E. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (page 22).**



Comments for pBAD/ThioE
4395 nucleotides

Arabinose O_2 operator region: bases 4-19
Arabinose O_1 operator region: bases 161-182
CAP binding site: bases 203-216
Arabinose I_1 and I_2 region: bases 213-251
Arabinose minimal promoter: bases 248-276
Ribosome binding site: 329-332
His Patch thioredoxin ORF: bases 346-674
Trx Forward priming site: bases 655-672
Enterokinase recognition site: bases 691-705
loxP site: bases 709-742
pBAD Reverse priming site: bases 795-812
rrnB transcriptional termination region: bases 845-1002
Ampicillin resistance gene: bases 1282-2142
pUC origin (pMB1-derived): 2287-2960
AraC ORF: bases 3491-4369 (complement)

continued on next page

Map and Features of pBAD/Thio-E, continued

Features of pBAD/Thio-E

The important elements of pBAD/Thio-E (4395 bp) are described in the following table. All features have been functionally tested. For more information on the regulation of gene expression by arabinose, see page 21.

Feature	Benefit
araBAD promoter (P_{BAD})	Provides tight, dose-dependent regulation of heterologous gene expression (Guzman et al., 1995).
O_2 region	Binding site of AraC that represses transcription from P_{BAD}.
O_1 region	Binding site of AraC that represses transcription of the araC promoter (PC) (transcribed on the opposite strand).
CAP binding site	Site where CAP (cAMP binding protein) binds to activate transcription from P_{BAD} and P_C.
I_2 and I_1 regions	Binding sites of AraC that activate transcription from P_{BAD}.
-10 and -35 regions	Binding sites of RNA polymerase for transcription from P_{BAD}.
Optimized ribosome binding site	Increases efficiency of recombinant fusion protein expression.
HP-thioredoxin	Provides a highly efficient fusion partner for translation of the fusion protein.
loxP	Site of recombination between the donor vector and pBAD/Thio-E (Hoess et al., 1982).
rrnB transcription termination region	Strong transcription termination region.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	High-copy replication and maintenance in *E. coli*.
araC gene	Encodes the regulatory protein for tight regulation of the P_{BAD} promoter (Lee, 1980; Lee et al., 1987; Schleif, 1992).

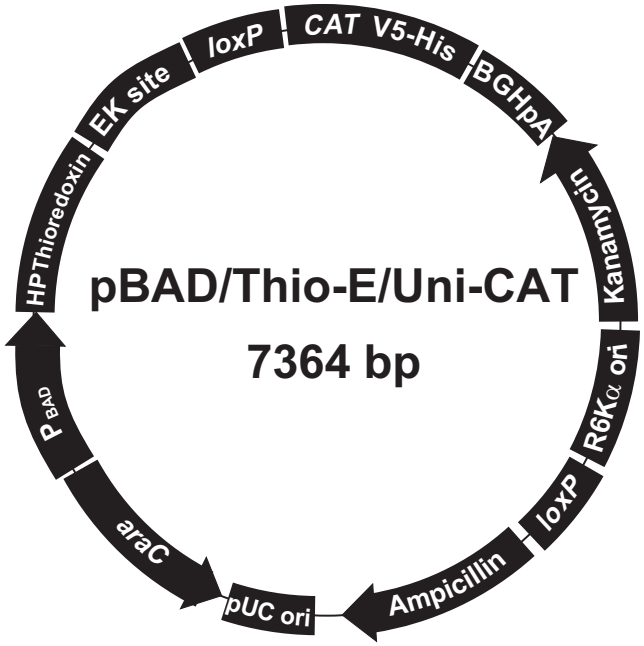
Map of pBAD/Thio-E/Uni-CAT

Description

pBAD/Thio-E/Uni-CAT is a 7364 bp control vector expressing a 42 kDa HP-thioredoxin-CAT fusion protein. The CAT gene was amplified and TOPO® Cloned into pUni/V5-His/Gene-TOPO®. The resulting vector was recombined with pBAD/Thio-E using *cre* recombinase to create pBAD/Thio-E/Uni-CAT. **Note**: pUni/V5-His/Gene-TOPO® is similar to pUni/V5-His-TOPO® except that it contains additional DNA between the TOPO® Cloning site and the V5 epitope.

Map of Control Vector

The figure below summarizes the features of the pBAD/Thio-E/Uni-CAT vector. **The complete nucleotide sequence for pBAD/Thio-E/Uni-CAT is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 22).**



Features of pBAD/Thio-E
7364 nucleotides

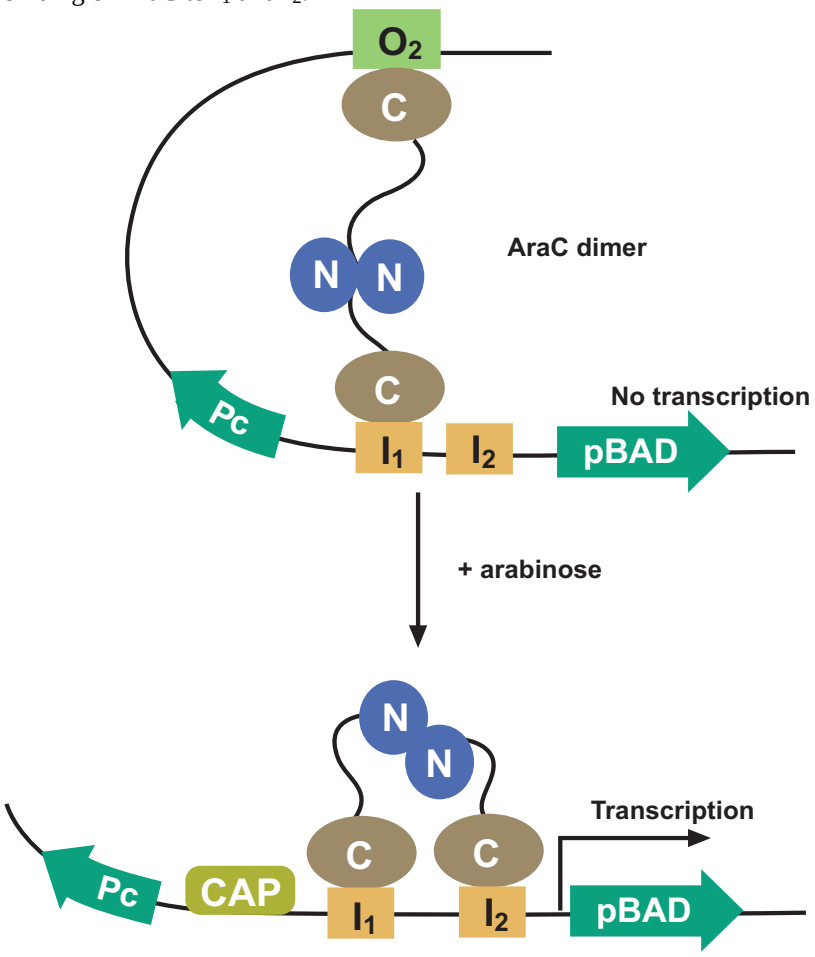
Arabinose O_2 operator region: bases 4-19
Arabinose O_1 operator region: bases 161-182
CAP binding site: bases 203-216
Arabinose I_1 and I_2 region bases 213-251
Arabinose minimal promoter: bases 248-276
Ribosome binding site: 329-332
His Patch thioredoxin ORF: 346-674
Trx Forward priming site: 655-672
Enterokinase recognition site: 691-705
loxP site: 709-742
CAT ORF: 763-1419
V5 epitope: 1432-1473
6xHis tag: 1483-1500
Uni1 Reverse priming site: 1562-1583
BGH polyadenylation sequence: 1581-1789
T7 transcription termination region: 1804-1932
Kanamycin resistance gene (C): 2905-2111
kan promoter (C): 3043-2906
R6K gamma origin: 3266-3657
Uni1 Forward priming site: 3625-3643
loxP site: 3678-3711
pBAD Reverse priming site: 3764-3781
rrnB transcriptional termination region: 3814-3971
Ampicillin resistance gene: 4251-5111
pUC origin: 5256-5929
AraC ORF (C): 7338-6460

Regulation by Arabinose

Regulation of the pBAD Promoter

The *ara*BAD promoter used in pBAD/Thio-E is both positively and negatively regulated by the product of the *ara*C gene (Ogden *et al.*, 1980; Schleif, 1992). AraC is a transcriptional regulator that forms a complex with L-arabinose. In the absence of arabinose the AraC dimer contacts the O_2 and I_1 half sites of the *ara*BAD operon, forming a 210 bp DNA loop (see the figure below). For maximum transcriptional activation two events are required.

- Arabinose binds to AraC and causes the protein to release the O_2 site and bind the I_2 site which is adjacent to the I_1 site. This releases the DNA loop and allows transcription to begin.
- The cAMP activator protein (CAP)-cAMP complex binds to the DNA and stimulates binding of AraC to I_1 and I_2.



Glucose Repression

Basal expression levels can be repressed by introducing glucose to the growth medium. Glucose acts by lowering cAMP levels, which in turn decreases the binding of CAP. As cAMP levels are lowered, transcriptional activation is decreased.

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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