USER GUIDE

invitrogen™
by life technologies™

pBAD/gIII A, B, and C

Vectors for Regulated, Secreted Expression of Recombinant Proteins Containing C-Terminal 6xHis Tags in *E. coli*

Catalog number V450-01

Revision date 2 March 2012

Publication Part number 25-0207

MAN0000058

life technologies™

Contents

Kit Contents and Storage

Shipping and Storage

Kits are shipped at room temperature. Upon receipt, store the plasmids and the 20% L-arabinose solution at –20°C. Store stabs 4°C.

Kit Contents

This kit contains the following items:

Contents	Cat. No.
20 µg each pBAD/gIII A, B, and C, at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL.	V450-01
20 µg pBAD/gIII/calmodulin at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL.	
1 mL sterile, 20% L-arabinose	
1 stab LMG194	
1 stab TOP10	

Note

The *E. coli* stabs supplied with the kit are guaranteed until the expiration date marked on tube when stored at 4°C. We recommend you prepare a set of glycerol master stocks prior to using your *E. coli* cells.

For research use only. Not intended for any human or animal therapeutic or diagnostic use.

Introduction

Product Overview

Description of the System

The pBAD/gIII plasmids are pBR322-derived expression vectors designed for regulated, secreted recombinant protein expression and purification in *E. coli*. The gene III signal sequence is utilized for secretion of the recombinant protein into the periplasmic space. Optimum levels of secreted, recombinant protein are possible using the *ara*BAD promoter (PBAD) from *E. coli*. The regulatory protein, AraC, is provided on pBAD/gIII vectors allowing regulation of PBAD.

Gene III Secretion Signal

Gene III encodes pIII, one of the minor capsid proteins from the filamentous phage fd (similar to M13 and f1). pIII is synthesized with an 18 amino acid, amino terminal signal sequence and requires the bacterial Sec system for insertion into the membrane (Boeke and Model, 1982; Boeke *et al.*, 1982; Davis *et al.*, 1985; Rapoza and Webster, 1993). The signal sequence is removed after crossing the inner membrane, and most proteins will be retained in the periplasmic space.

Regulation of Expression by L-arabinose

In the presence of L-arabinose, expression from PBAD is turned on while the absence of L-arabinose produces very low levels of transcription from PBAD (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, thus lowering expression of the catabolite-repressed PBAD promoter (Miyada et al., 1984). By varying the concentration of L-arabinose, protein expression levels can be manipulated to optimize expression of soluble, secreted protein. In addition, the tight regulation of PBAD 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, refer to Schleif, 1992.

Experimental Outline

The table below describes the basic steps needed to clone and express your protein using pBAD/gIII. For more details, refer to the page(s) indicated.

Step	Action	Page
1	Develop a cloning strategy to ligate your gene of interest into pBAD/gIII A, B, or C.	4
2	Propagate and maintain the empty vectors by transforming them into a *rec*A, *end*A *E. coli* host (i.e. TOP10).	3
3	Ligate your gene of interest into pBAD/gIII, transform into TOP10 or LMG194, and select on 50–100 µg/mL ampicillin.	4–8
4	Sequence your construct to ensure that it is in frame with the C-terminal peptide if you elect to create a fusion protein.	8
5	Perform a 4-hour expression using a 10,000-fold range of L-arabinose concentrations (e.g. 0.00002%, 0.0002%, 0.002%, 0.02%, and 0.2%).	9–10
6	Optimize expression by varying L-arabinose concentration or the time of induction.	11
7	Purify your recombinant protein by chromatography on metal-chelating resin (e.g. ProBond™).	13–16

Detection of Recombinant Proteins

Expression of your recombinant protein can be detected using an antibody to the appropriate epitope. The table below describes the antibodies available for use with pBAD/gIII (see page 23 for ordering). Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods.

Vector	Epitope	Antibody
pBAD/gIII	*c-myc*	Anti-*Myc*
		Anti-*Myc*-HRP
	C-terminal polyhistidine tag	Anti-His(C-term)
		Anti-His(C-term)-HRP

Purification of Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple, easy purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using ProBond™ Resin. To purify proteins expressed using pBAD/gIII, the ProBond™ Purification System or the ProBond™ resin in bulk are available separately. See page 23 for ordering information.

Methods

General Cloning

Introduction

The following information is provided to help you clone your gene of interest into pBAD/gIII. For basic information on DNA ligations, E. coli transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, see Molecular Cloning: A Laboratory Manual (Sambrook et al., 1989) or Current Protocols in Molecular Biology (Ausubel et al., 1994).

E. coli Host

For cloning and transformation, we recommend using a *rec*A, *end*A strain such as TOP10 (included in the kit). This strain is capable of transporting L-arabinose, but not metabolizing it. This is important for expression studies as the level of L-arabinose will be constant inside the cell and not decrease over time. Note that other strains may be suitable for general use. Be sure to check the genotype of your strain. It should be araBADC- and araEFGH+ (Bachmann, 1990).

The *E. coli* strain LMG194 (Guzman et al., 1995) is included in the kit to allow additional repression for low basal level expression of toxic genes. This strain is capable of growth on minimal medium (RM medium), which allows repression of PBAD by glucose. **Once you have determined that you have the correct construct, transform it into LMG194 prior to performing expression experiments.**

For your convenience, TOP10 is available as electrocompetent or chemically competent cells in a One Shot® kit format (see page 23 for ordering).

Genotype of TOP10

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara*A-*leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G.

Note: This strain is *ara*BADC-. It is deleted for both *ara*BA and *ara*C, and the gene for *ara*D has a point mutation in it, making it inactive.

Genotype of LMG194

F- Δ*lac*X74 *gal* E *thi rps*L Δ*pho*A (*Pvu* II) Δ*ara*714 *leu*::Tn*10*.

Note: This strain is deleted for araBADC. It is also streptomycin and tetracycline resistant.

Maintenance of pBAD/gIII

To propagate and maintain pBAD/gIII, use the supplied 0.5 µg/µL stock solution in TE, pH 8.0 to transform a *rec*A, *end*A *E. coli* strain like DH5α™, TOP10 or equivalent. Transformants are selected on LB plates containing 50–100 µg/mL ampicillin. **Note**: Strains like DH5α™ may be used **only** for propagation of pBAD/gIII, but not expression of recombinant proteins (see explanation above).

Cloning into pBAD/gIII

Important

To generate secreted, recombinant proteins that are expressed correctly and contain the C-terminal fusion peptide, it is necessary to clone in frame with **BOTH** the gene III secretion signal and the C-terminal peptide. The initiation ATG of the secretion signal is correctly spaced from the optimized RBS to ensure optimal translation.

To facilitate cloning, the pBAD/gIII vector is provided in three different reading frames. They differ only in the spacing between the signal sequence and the multiple cloning site. For proper expression, first determine which restriction sites are appropriate for ligation and then which vector will preserve the reading frame at **BOTH** the 5´ and the 3´ ends. You may have to use PCR to create a fragment with the appropriate restriction sites to clone in frame at both ends. Be sure that there is no stop codon in the open reading frame of your gene (except as noted below).

Note

If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon at the end of your gene.

pBAD/gIII Multiple Cloning Site

The multiple cloning sites of each version of pBAD/gIII are provided on the following pages. Restriction sites are labeled to indicate cleavage site. The boxed sequence is the variable region that facilitates in-frame cloning with the gene III signal sequence.

Features of the *ara*BAD and *ara*C promoters are marked and described as follows. For more information see Lee, 1980; Miyada, *et al.*, 1984; Lee, *et al.*, 1987; and Schleif, 1992.

- 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 *ara*C promoter (P_C) (transcribed on the opposite strand; not shown).
- CAP binding site: Site where CAP (cAMP binding protein) binds to help 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 of P_{BAD}.

Each multiple cloning site has been confirmed by sequencing and functional testing.

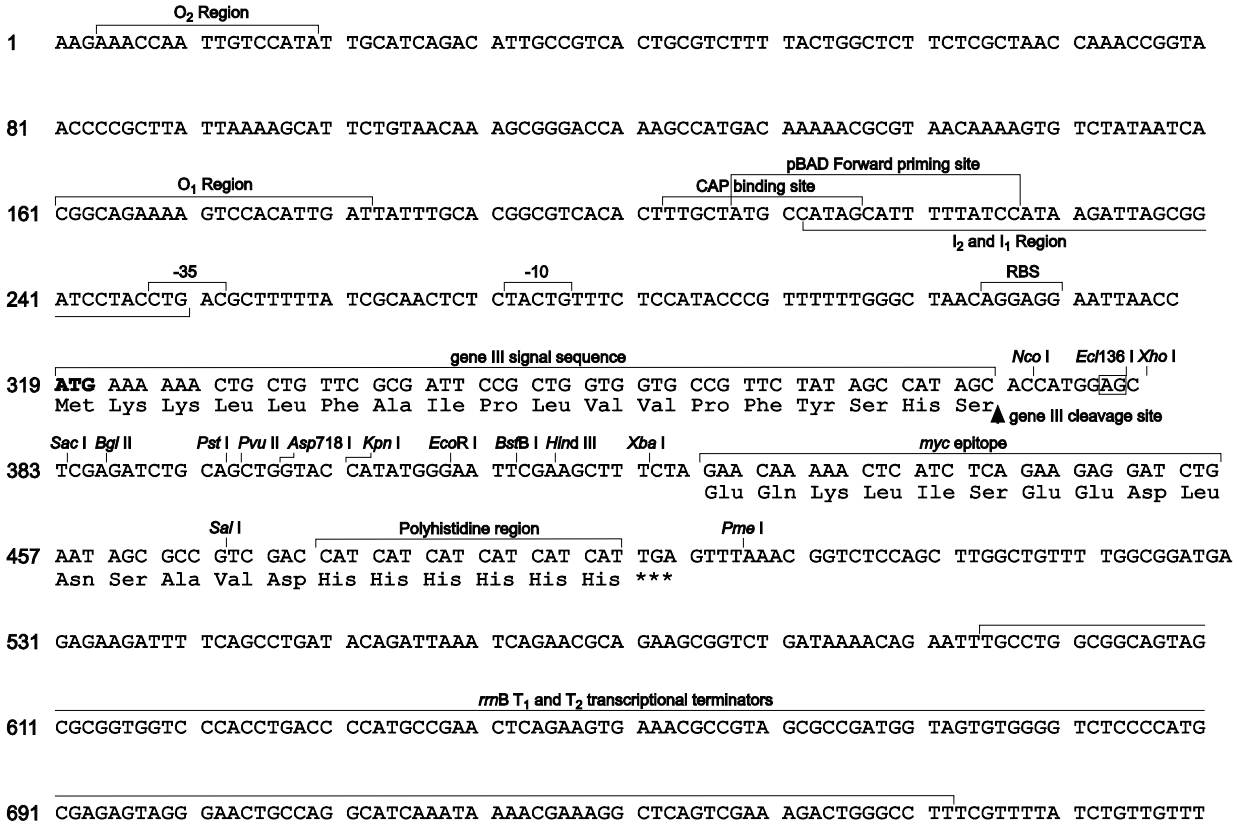
Cleavage of the Gene III Signal

Cleavage of the gene III signal occurs after Ser-His-Ser (see the multiple cloning site diagrams on the following pages). To minimize the number of additional amino acids at the N-terminus of your protein, we recommend using the *Nco* I site to clone your gene of interest closest to the gene III signal sequence. If you use the *Nco* I site, correct cleavage of the gene III will leave a threonine in front of the methionine.

Continued on next page

Cloning into pBAD/gIII, Continued

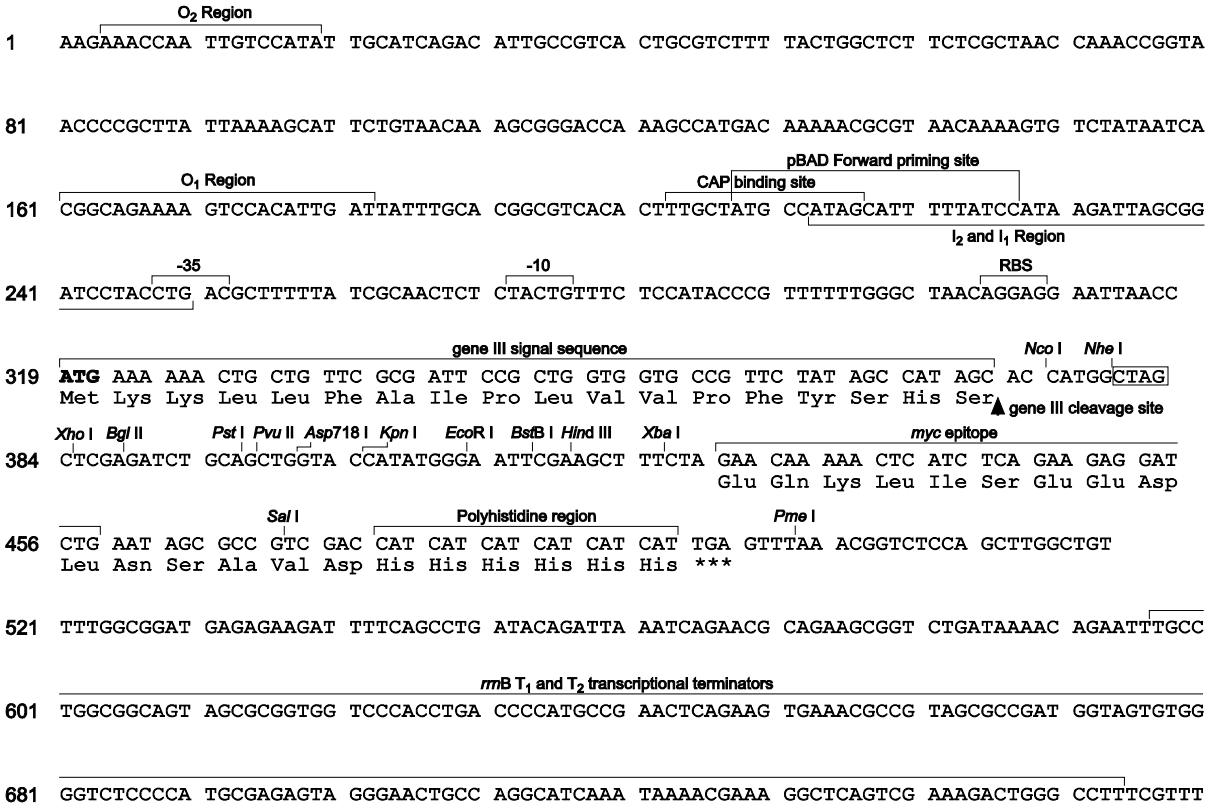
pBAD/gIII A



Continued on next page

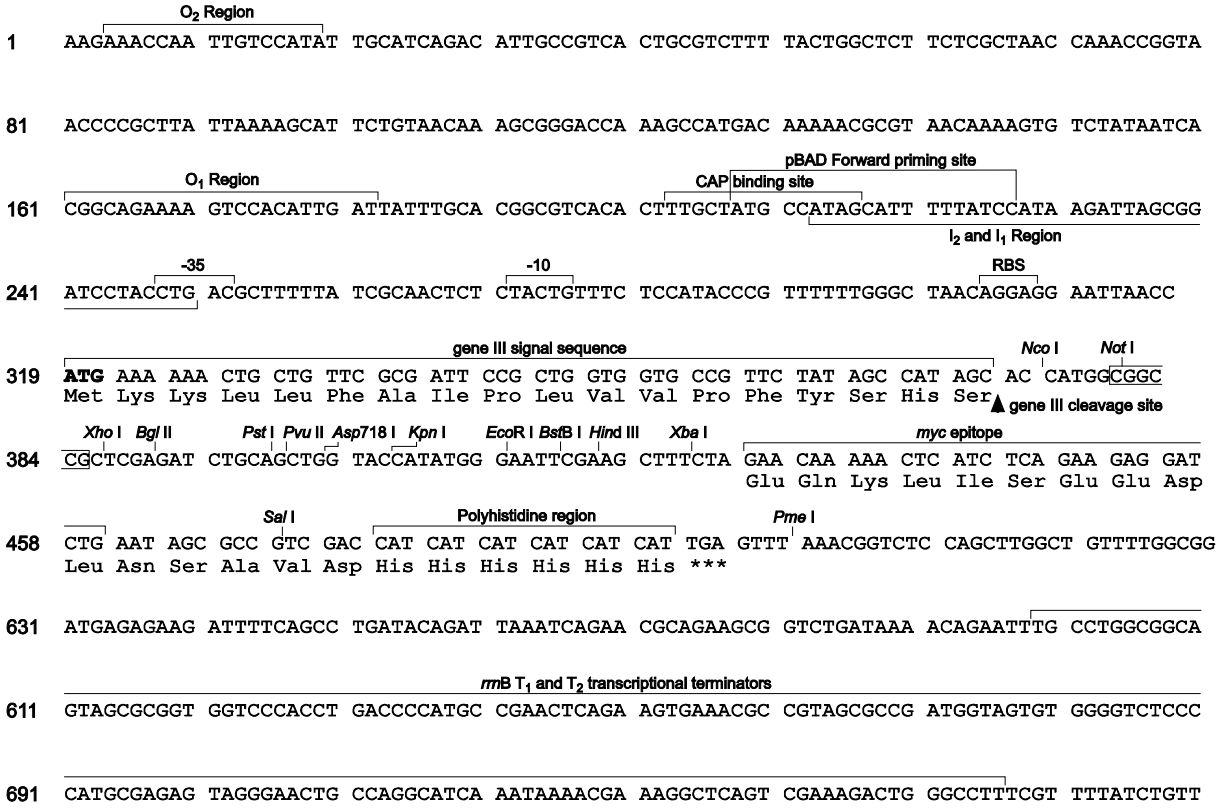
Cloning into pBAD/gIII, Continued

pBAD/gIII B



Cloning into pBAD/gIII, Continued

pBAD/gIII C



E. coli Transformation

E. coli Transformation

After ligating your insert into the appropriate vector, transform your ligation mixtures into TOP10 cells and select on LB plates containing 50–100 µg/mL ampicillin. Select 10–20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct to confirm that your gene is in frame with the appropriate tag.

Glycerol Stock

Once you have obtained your desired construct, we recommend that you store your clone as a glycerol stock.

1. Grow 1–2 mL of the strain containing your construct in pBAD/gIII to log phase (OD_{600} = 0.5–0.7) in LB containing 50–100 µg/mL ampicillin.
2. Combine 0.85 mL of the culture with 0.15 mL of sterile glycerol.
3. Mix the solution by vortexing.
4. Transfer to an appropriate vial for freezing and cap.
5. Freeze in an ethanol/dry ice bath or liquid nitrogen and then transfer to -70°C for long-term storage.

Expression

Introduction

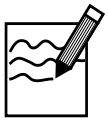
Since each recombinant protein has different characteristics that may affect optimum expression, it is helpful to vary the L-arabinose concentration and/or run a time course of expression to determine the best conditions for optimal expression of your particular protein. A mock expression consisting of the pBAD/gIII vector alone should be done as a negative control. pBAD/gIII/calmodulin is included for use as a positive expression control (see page 19). TOP10 may be used as a general host for expression. LMG194 should be used if your protein is toxic or essential to *E. coli*.

Basic Strategy

We recommend that you check for expression of your protein first, then check for solubility and secretion. Use the following strategy to determine the optimal expression level.

1. **Pilot Expression.** In this expression experiment you will vary the amount of L-arabinose over a 10,000-fold range (0.00002% to 0.2%) to determine the approximate amount of L-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 L-arabinose concentrations spanning the amount determined in Step 1. Or you may wish to perform a time course.

Note: If you transformed your pBAD/gIII construct into LMG194, be sure to perform your expression experiments in RM medium with glucose (see page 21 for recipe) to ensure low basal levels of your protein.



Note

Expression of your protein with the C-terminal tag will increase the size of your protein by ~2 kDa. Be sure to account for any additional amino acids between the tag and your protein.

Materials Needed

- SOB or LB containing 50 µg/mL ampicillin (see **Recipes**, pages 20)
- RM medium containing glucose (see **Recipes**, page 21)
- 37°C shaking incubator
- 20% L-arabinose (provided)
- 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
- 70°C water bath
- Lysis Buffer (see page 22 for recipe)
- Sterile water

Continued on next page

Pilot Expression

This experiment is designed to test for and optimize expression of your recombinant protein. **Remember to include a negative control (cells only or cells containing the empty vector) and a positive control (pBAD/gIII/calmodulin) to evaluate your expression experiment.**

1. For each transformant or control, inoculate 2 mL of SOB or LB containing 50 µg/mL ampicillin with a single recombinant *E. coli* colony. **Note: If you are using LMG194 as a host, use RM medium containing glucose and 50-100 µg/mL ampicillin at all steps.**
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 medium containing 50 µg/mL ampicillin.
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% L-arabinose with sterile water (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.
9. Add L-arabinose to the five 10 mL cultures as follows:

Tube	Volume (mL)	Stock Solution	Final Concentration
1	0.1	0.002%	0.00002%
2	0.1	0.02%	0.0002%
3	0.1	0.2%	0.002%
4	0.1	2%	0.02%
5	0.1	20%	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. In general, four hours is sufficient for expression of most proteins. You may discard the remaining culture or you may continue on with the time course.

Continued on next page

Preparing Samples

Before starting, prepare SDS-PAGE gels to analyze all the samples you collected. **Note**: If you already know that your protein is insoluble, use the protocol on the next page to analyze your samples.

1. When all the samples have been collected from Steps 8 and 11, previous page, resuspend each pellet in 100 µL of 1X SDS-PAGE sample buffer.
2. Heat 5 minutes at 70°C and centrifuge briefly.
3. Load 5 µL of each sample on an SDS-PAGE gel and electrophorese. Save your samples by storing at –20°C.

Sample Analysis

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 a negative control (empty vector) to distinguish recombinant proteins from background proteins.
3. Use the positive control (pBAD/gIII/calmodulin) to confirm that growth and induction was done properly. The positive control should yield a 30 kDa protein.
4. You should be able to determine the approximate L-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 the Anti-*Myc* antibodies to detect expression of your protein. **Note**: Proteins expressed using pBAD/gIII may also be detected with the Anti-His (C-term) Antibody which recognizes histidine tags with a free carboxyl group (see page 23 for ordering).

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

Optimizing Expression

Once you have detected expression of your protein, you may wish to perform some experiments to further optimize expression. Use the Pilot Expression protocol, but vary the L-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 5–6 hour time period, taking time points every hour, to determine if varying the time increases expression.

Remember to store your time points at –20°C.

If your protein is insoluble, you may wish to analyze the supernatant and pellet of lysed cells when you vary the L-arabinose concentration. Refer to the protocol on the next page to prepare samples.

Continued on next page

Insoluble Proteins

If you suspect that your protein may be insoluble, analyze your expression samples using the following protocol.

1. When all the samples have been collected, thaw and resuspend each pellet in 100 µL of Lysis Buffer (see **Recipes**, page 22).
2. Incubate on ice for 30 minutes.
3. Perform 3 cycles of freeze-thaw (freeze in liquid nitrogen or a dry ice bath, then thaw at 42°C).
4. Incubate at 37°C for 30 minutes.
5. Centrifuge samples to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
6. Mix together equal amounts of supernatant and 2X SDS Sample buffer and heat for 5 minutes at 70°C.
7. Add 200 µL of 1X SDS-PAGE sample buffer to pellets from Step 5 and heat 5 minutes at 70°C.
8. Load 10 µL of the supernatant sample and 10 µL of the pellet sample onto an SDS-PAGE and electrophorese.
9. Analyze for optimal, soluble expression of your protein. Ideally, most of your recombinant protein should be soluble. If the majority of your protein is still insoluble, try the induction at 28°C or 30°C instead of 37°C. Expressing at a lower temperature may help proteins fold correctly.

Analyzing Secreted Protein

The gene III signal sequence is designed to direct your protein to the periplasmic space. To test for secretion, you will need to osmotically shock your cells to release the recombinant protein from the periplasmic space. Refer to the protocol on pages 13–15 for details.

Expressing Toxic Proteins

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

1. Transform your construct into LMG194. LMG194 can be grown in RM medium, which enables repression of P_{BAD} by glucose.
2. Follow the Pilot Expression on page 10, substituting RM Medium + Glucose medium (see page 21) to grow the cells.
3. Be sure to monitor the OD_{600} as the cells will grow more slowly in RM medium.
4. Induce with various concentrations of L-arabinose as described in the Pilot Expression.
5. Monitor OD_{600} over time be sure cells are growing.

Osmotic Shock

Introduction

The procedure below may be used to check for secretion of your recombinant protein and/or partial purification of your recombinant protein. The positive control vector pBAD/gIII/calmodulin may be used to evaluate secretion and the osmotic shock procedure.

Before Starting

- SOB or LB containing 50 µg/mL ampicillin
- Osmotic Shock Solution 1 (see page 22)
- Osmotic Shock Solution 2 (see page 22)

Control

pBAD/gIII/calmodulin is provided as a positive control for osmotic shock. About 50% of the total expressed calmodulin (~0.2 mg/mL) can be recovered in the shock fluid after osmotic shock.

Note

Osmotic shock works best on fresh cells. Do not store cells before shocking. Do not shock frozen cells.

Growth and Induction of Cells

Use the conditions you developed in the **Expression** section to grow and induce expression of your protein.

1. Inoculate 2 mL of SOB or LB containing 50 µg/mL ampicillin with a single recombinant *E. coli* colony. **Note: If you are using LMG194 as a host, use RM medium containing glucose and 50–100 µg/mL ampicillin.**
2. Grow overnight at 37°C with shaking (225–250 rpm) to OD_{600} = 1–2.
3. The next day, inoculate 10–25 mL of SOB or LB containing 50 µg/mL ampicillin with 0.1–0.25 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). Record the OD_{600}.
5. Remove a 1 mL sample of cells, centrifuge at maximum speed in a microcentrifuge for 30 seconds, aspirate the supernatant, **and store the cells on ice. Do not freeze the cells. This is the zero time point sample.**
6. Add the appropriate amount of L-arabinose determined previously to induce expression of your protein.
7. Grow the cells to the optimal time point as previously determined. Read and record the OD_{600}.
8. Remove a 1 mL sample, centrifuge as described in Step 5, aspirate the supernatant, **and store the cells on ice.**
9. Take the rest of the culture and discard. Do not save the cells. Proceed to the next section.

Continued on next page

Osmotic Shock

1. Resuspend cell pellets from **Growth and Induction of Cells**, Steps 5 and 8 (previous page) in Osmotic Shock Solution 1 (with sucrose) to an OD_{600} of 5.0. Use the OD_{600} value you recorded for each time point to determine in what volume you should resuspend the cells.

   Formula: $V_R = (OD_{600}$ of sample/5.0) × V_S

   Where V_R is the volume to resuspend the cell pellet and V_S is the original sample volume of the cell suspension

   Example: If a 1 mL sample of your cells has an OD_{600} of 0.5 for the zero time point, then:

   $V_R = (0.5/5.0) \times 1$ mL = 0.1 mL or 100 µL. This is the volume in which to resuspend your cells. Note that each pellet may need to be resuspended in a different volume.
2. Incubate cells on ice for 10 minutes. Centrifuge for 1 minute at 4°C and decant the supernatant.
3. Resuspend cell pellets in Osmotic Shock Solution 2 using the same volumes from Step 1. Incubate on ice for 10 minutes.
4. Centrifuge for 10 minutes at 4°C. Transfer the supernatant (shock fluid) to a clean tube and keep on ice.
5. Resuspend the pellets from Step 4 in the same volume of Osmotic Shock Solution 2 as was used in Step 1. Note that each pellet may be resuspended in a different volume.
6. You now have four samples – a supernatant (shock fluid) and a pellet sample (cells) for the zero time point and a supernatant and pellet sample for the optimal time point. If you included the positive control, you will have four more samples for a total of eight.
7. These samples may be frozen at –20°C if you do not want to run a gel the same day you prepare samples. Proceed to **Analysis of Osmotic Shock Samples** on the next page.

Continued on next page

Analysis of Osmotic Shock Samples

1. The samples from **Osmotic Shock**, step 6 (previous page) are analyzed on an SDS-PAGE gel. Use 10 µL aliquots for each sample. Prepare and load the samples onto the gel so you can compare shock fluid with cells for each time point. Run the gel and process.
2. Use the following table to evaluate your experiment:

IF the sample containing calmodulin ...	AND the sample containing the fusion protein ...	THEN...
is in the supernatant fraction	is also in the supernatant fraction	the fusion protein is secreted and released by osmotic shock. Osmotic shock can be used as a purification step.
is in the supernatant fraction	is in the pellet fraction	the fusion protein is either not secreted or is not released by osmotic shock. See next section below.
is in the pellet fraction	is also in the pellet fraction	review the osmotic shock procedure and make sure the correct buffers were used in the correct order. If the osmotic shock step was done properly, calmodulin should be in the supernatant.

Recombinant Protein Appears not to be Secreted

It may happen that because of the nature of your protein, it is not properly secreted or shocked out. The protein may be retained in the cytoplasm or associated with the inner cell membrane. You may need to prepare a whole cell lysate prior to purifying your protein. For additional information, refer to *Guide to Protein Purification*, pages 147–153 (Deutscher, 1990).

Note: If the recombinant protein is retained in the cytoplasm, the gene III signal sequence will not be removed from the protein.

Activity Assay

If your fusion protein was successfully purified by osmotic shock, you may wish to assay for the activity of your desired protein. If the fusion protein retains significant levels of activity, you may scale-up your purification to produce more fusion protein.

Purification

Scale-up of Expression for Purification

Use the conditions determined in the previous section to grow and induce 50 mL of cells. This is the largest culture volume to use with the 2 mL prepacked columns included in the ProBond™ System. If you need to purify larger amounts of recombinant protein, you may need more ProBond™ resin. See page 23 for ordering information. **Note**: Remember to use RM medium (page 21) with LMG194.

1. Inoculate 2 mL of SOB or LB containing 50 µg/mL ampicillin 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 ampicillin 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 L-arabinose to induce expression.
6. Grow at 37°C with shaking until the optimal time point is reached. Harvest the cells by centrifugation (3000 × *g* for 10 minutes at 4°C).
7. At this point, you may proceed directly to purification (ProBond™ Purification System manual) or store at –80°C for future use.

Purification

For help with purification of your recombinant protein, refer to the ProBond™ Purification System manual.

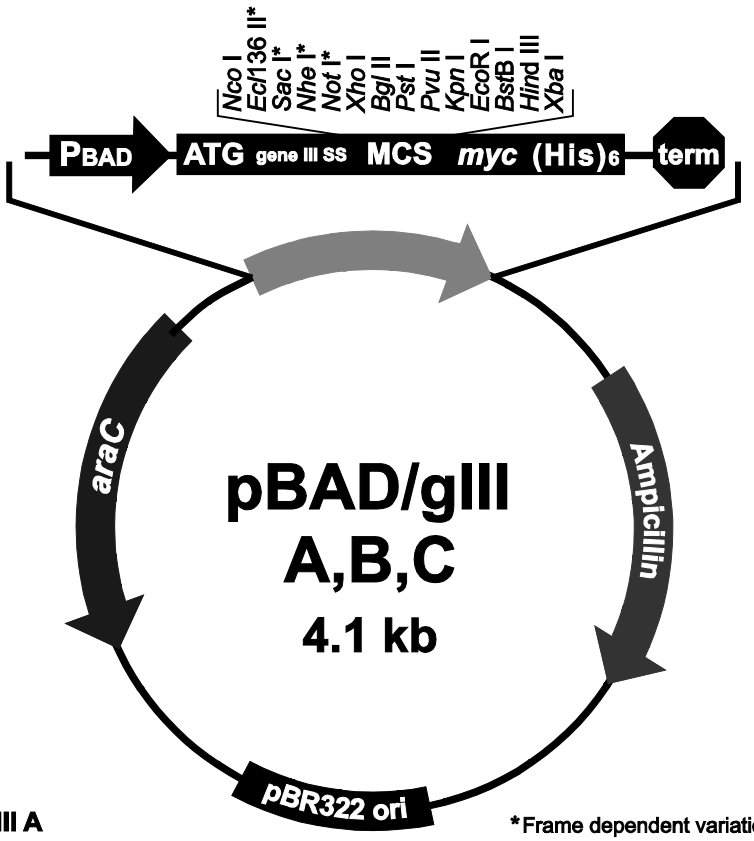
If you are using another type of resin, refer to the manufacturer's recommendations.

Appendix

pBAD/gIII Vector

Map of pBAD/gIII

The figure below summarizes the features of the pBAD/gIII vector. Vector sequences for all three pBAD/gIII vectors can be downloaded from www.lifetechnologies.com or by contacting Technical Support (see page 24). Details of each multiple cloning site are shown on pages 5–7.



Comments for pBAD/gIII A
4145 nucleotides

*ara*BAD promoter region: bases 4-276
Initiation ATG: bases 319-321
Gene III secretion signal: bases 319-373
Multiple cloning site: bases 374-428
myc epitope: bases 427-456
Polyhistidine region: bases 472-489
*rrn*B transcriptional termination region: bases 595-752
Ampicillin resistance gene (ORF): bases 1032-1892
pBR322 origin: bases 2037-2710
AraC ORF: bases 3241-4139 (opposite strand)

*Frame dependent variations.
Version A contains *Sac* I and *Ecl*136 II only.
Version B contains *Nhe* I only.
Version C contains *Not* I only.

Features of pBAD/gIII

The important elements of pBAD/gIII A (4145 bp), pBAD/gIII B (4147 bp), and pBAD/gIII C (4149 bp) are described in the following table. All features have been functionally tested.

Feature	Benefit
*ara*BAD promoter (P_{BAD})	Provides tight, dose-dependent regulation of heterologous gene expression (Guzman *et al.*, 1995)
Optimized ribosome binding site	Increases efficiency of recombinant fusion protein expression
Initiation ATG	Provides a translational initiation site for the fusion protein
Gene III secretion signal	Permits secretion of recombinant protein into the periplasmic space (Rapoza and Webster, 1993)
Multiple cloning site	Allows insertion of your gene for expression
C-terminal *myc* epitope tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of the fusion protein by the Anti-*Myc* Antibody (see page 23) (Evans *et al.*, 1985)
C-terminal polyhistidine region	Forms metal-binding site for affinity purification of recombinant fusion protein on metal-chelating resin (i.e. ProBond™) In addition, it allows detection of the recombinant protein with Anti-His (C-term) Antibody (see page 2)
*rrn*B transcription termination region	Strong transcription termination region
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*
pBR322 origin	Low copy replication and growth in *E. coli*
*ara*C gene	Encodes the regulatory protein for tight regulation of the P_{BAD} promoter (Lee, 1980; Schleif, 1992)

Continued on next page

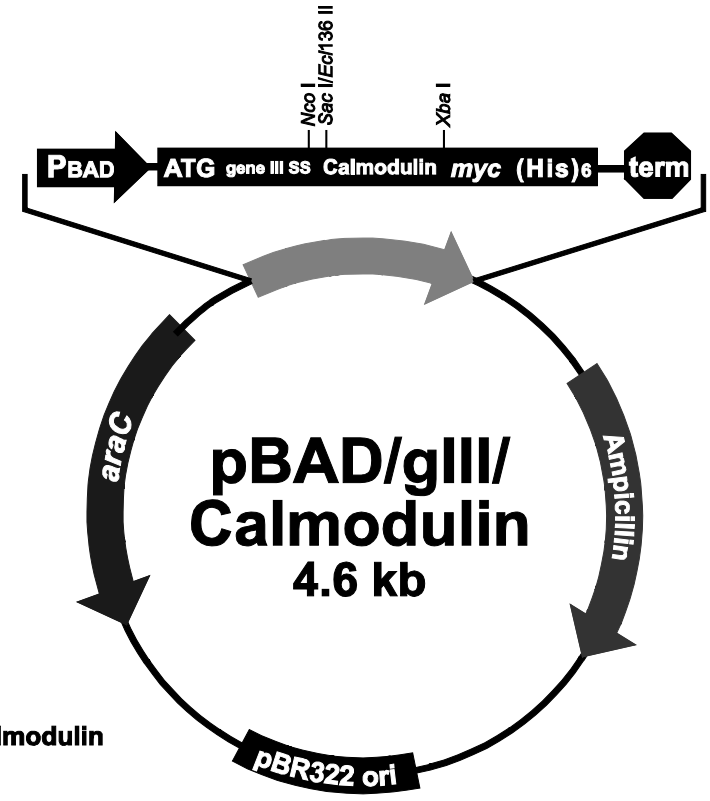
Map of pBAD/gIII/calmodulin

Description

pBAD/gIII is a 4556 bp control vector containing the gene for calmodulin fused to the C-terminal peptide. It was constructed by digesting pBAD/gIII A with *Sac* I and *Xba* I, and ligating an 455 bp *Sac* I-*Xba* I fragment containing the calmodulin gene. The calculated molecular weight of calmodulin fused to the gene III signal sequence is 21.3 kDa. The observed molecular weight from an SDS-PAGE gel is 25–30 kDa.

Map of Control Vector

The figure below summarizes the features of the pBAD/gIII/calmodulin vector. The nucleotide sequence for pBAD/gIII/calmodulin may be downloaded from www.lifetechnologies.com or by contacting Technical Support (see page 24).



Comments for pBAD/gIII/calmodulin
4556 nucleotides

*ara*BAD promoter region: bases 4-276
Initiation ATG: bases 319-321
Gene III secretion signal: bases 319-373
Calmodulin ORF: bases 385-826
myc epitope: bases 838-867
Polyhistidine region: bases 883-900
*rrn*B transcriptional termination region: bases 1006-1163
Ampicillin resistance gene (ORF): bases 1443-2303
pBR322 origin: bases 2448-3121
AraC ORF: bases 3649-4530 (opposite strand)

Recipes

Low Salt LB Medium (with Ampicillin)

LB Medium (per liter)

1% Tryptone

0.5% Yeast Extract

0.5% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with 5 M NaOH and bring the volume to 1 liter.
3. Autoclave for 20 minutes on liquid cycle.
4. Let solution cool to ~55°C. Add ampicillin to a final concentration of 50 µg/mL. Store the medium at 4°C. **Medium is stable for only 1–2 weeks.**

Low Salt LB Agar Plates with Ampicillin

LB Medium (per liter)

1% Tryptone

0.5% Yeast Extract

0.5% NaCl

1.5% Agar

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with 5 M NaOH, add 15 g agar, and bring the volume to 1 liter.
3. Autoclave for 20 minutes on liquid cycle.
4. Let agar cool to ~55°C. Add ampicillin to a final concentration of 50 µg/mL.
5. Pour into 10 cm petri plates. Let the plates harden, then invert and store at 4°C. **Plates containing ampicillin are stable for 1–2 weeks.**

Continued on next page

SOB Medium (with Ampicillin)

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 ampicillin to 50 µg/mL.
5. Store at 4°C. **Medium is stable for only 1–2 weeks.**

RM Medium + Glucose

1X M9 Salts (See below for recipe for 10X M9 Salts)

2% Casamino Acids

0.2% glucose

1 mM MgCl2

50–100 µg/mL ampicillin

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 MgCl2	1 mL
20% glucose	10 mL
100 mg/mL ampicillin	0.5 to 1 mL

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

Continued on next page

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. Add 1 mL of 1 M thiamine (filter-sterilize) per 1 L 1X M9 medium.
4. Store at room temperature.

Lysis Buffer

10 mM Tris-HCl, pH 8

1 mM EDTA

0.5 mg/mL lysozyme

0.1 mg/mL DNase I

10 mM $CaCl_2$

1. Prepare just before use. Take 10 mL of TE buffer and add 5 mg of lysozyme, 1 mg of DNase I, and 0.1 mL of 1 M $CaCl_2$.
2. Gently mix and store on ice. Use immediately.

Osmotic Shock Solution 1

20 mM Tris-HCl, pH 8

2.5 mM EDTA

20% Sucrose

1. For 1 liter, combine 200 g sucrose, 20 mL 1 M Tris-HCl pH 8.0, and 5 mL 0.5 M EDTA and bring up to a final volume of 1 liter with water.
2. Stir to dissolve sucrose.
3. Autoclave or filter-sterilize.
4. Store at room temperature or at 4°C.

Osmotic Shock Solution 2

20 mM Tris-HCl, pH 8

2.5 mM EDTA

1. For 1 liter, combine 20 mL 1 M Tris-HCl pH 8.0 and 5 mL 0.5 M EDTA and bring up to a final volume of 1 liter with water.
2. Autoclave or filter-sterilize.
3. Store at room temperature or at 4°C.

Accessory Products

Additional Products

Many products suitable for use with pBAD/gIII are available separately. Ordering information for these reagents is provided below.

Item	Quantity	Cat. no.
One Shot® TOP10 Electrocomp™ *E. coli*	21 × 50 µL	C4040-52
One Shot® TOP10 Chemically Competent *E. coli*	21 × 50 µL	C4040-03

Detection of Recombinant Fusion Proteins

You can detect expression of your recombinant fusion protein from pBAD/gIII using the Anti-*Myc* and Anti-His antibodies available from Life Technologies.

Epitope	Antibody	Cat. no.
c-myc	Anti-*Myc*	R950-25
	Anti-*Myc*-HRP	R951-25
C-terminal polyhistidine tag	Anti-His(C-term)	R930-25
	Anti-His(C-term)-HRP	R931-25

Purification of Recombinant Protein

The presence of the polyhistidine tag in pBAD/gIII allows purification of your recombinant fusion protein using a nickel-charged agarose resin such as ProBond™. Ordering information is provided below.

Item	Quantity	Cat. no.
ProBond™ Nickel-Chelating Resin	50 mL 150 mL	R801-01 R801-15
ProBond™ Purification System	6 purifications	K850-01
Purification Columns (10 mL polypropylene columns)	50	R640-50

Technical Support

Obtaining support

For the latest services and support information for all locations, go to www.lifetechnologies.com/support.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited warranty

Life Technologies Corporation is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about a Life Technologies product or service, contact our Technical Support Representatives.

All Life Technologies products are warranted to perform according to specifications stated on the certificate of analysis. The Company will replace, free of charge, any product that does not meet those specifications. This warranty limits the Company's liability to only the price of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. The Company reserves the right to select the method(s) used to analyze a product unless the Company agrees to a specified method in writing prior to acceptance of the order.

Life Technologies makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore the Company makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, report it to our Technical Support Representatives.

Life Technologies Corporation shall have no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Limited Use Label License No: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License 6X His Tag

This product is licensed from Hoffmann-La Roche, 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 araB Promoter

Inquiries for any commercial use, including production of material to be sold commercially or used in production or in product development efforts, which includes efforts toward regulatory approval, should be made directly to Xoma Corporation, 2910 Seventh Street, Berkeley, CA 94710, Tel: 1-510-644-1170 Fax: 1-510-649-7571.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Bachmann, B. J. (1990). Linkage map of Escherichia coli K-12, edition 8. Microbiol. Rev *54*, 130-197.

Boeke, J. D., and Model, P. (1982). A Prokaryotic Membrane Anchor Sequence: Carboxyl Terminus of Bacteriophage f1 Gene III Protein Retains it in the Membrane. Proc. Natl. Acad. Sci. USA *79*, 5200-5204.

Boeke, J. D., Model, P., and Zinder, N. D. (1982). Effects of Bacteriophage f1 Gene III Protein on the Host Cell Membrane. Mol. Gen. Genet. *186*, 185-192.

Carson, M. J., Barondess, J. J., and Beckwith, J. (1991). The FtsQ Protein of *Escherichia coli*: Membrane Topology, Abundance, and Cell Division Phenotypes Due to Overproduction and Insertion Mutations. J. Bacteriol. *173*, 2187-2195.

Dalbey, R. E., and Wickner, W. (1985). Leader Peptidase Catalyzes the Release of Exported Proteins from the Outer Surface of the *Escherichia coli* Plasma Membrane. J. Biol. Chem. *260*, 15925-15931.

Davis, N. G., Boeke, J. D., and Model, P. (1985). Fine Structure of a Membrane Anchor Domain. J. Mol. Biol. *181*, 111-121.

Deutscher, M. P. (1990) Guide to Protein Purification. In *Methods in Enzymology*, Vol. 182. (J. N. Abelson and M. I. Simon, eds.) Academic Press, San Diego, CA.

Evans, G. I., Lewis, G. K., Ramsay, G., and Bishop, V. M. (1985). Isolation of Monoclonal Antibodies Specific for *c-myc* Proto-oncogene Product. Mol. Cell. Biol. *5*, 3610-3616.

Guzman, L.-M., Barondess, J. J., and Beckwith, J. (1992). FtsL, an Essential Cytoplasmic Membrane Protein Involved in Cell Division in *Escherichia coli*. J. Bacteriol. *174*, 7716-7728.

Guzman, L.-M., Belin, D., Carson, M. J., and Beckwith, J. (1995). Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose P_{BAD} Promoter. J. Bacteriol. *177*, 4121-4130.

Kuhn, A., and Wickner, W. (1985). Isolation of Mutants in M13 Coat Protein That Affect its Synthesis, Processing and Assembly into Phage. J. Biol. Chem. *260*, 15907-15913.

Lee, N. (1980) Molecular Aspects of *ara* Regulation. In *The Operon*, J. H. Miller and W. S. Reznikoff, eds. (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory), pp. 389-410.

Lee, N., Francklyn, C., and Hamilton, E. P. (1987). Arabinose-Induced Binding of AraC Protein to *ara*I_2 Activates the *ara*BAD Operon Promoter. Proc. Natl. Acad. Sci. USA *84*, 8814-8818.

Miyada, C. G., Stoltzfus, L., and Wilcox, G. (1984). Regulation of the *ara*C Gene of *Escherichia coli*: Catabolite Repression, Autoregulation, and Effect on *ara*BAD Expression. Proc. Natl. Acad. Sci. USA *81*, 4120-4124.

Rapoza, M. P., and Webster, R. E. (1993). The Filamentous Bacteriophage Assembly Proteins Require the Bacterial SecA protein for Correct Localization to the Membrane. J. Bacteriol. *175*, 1856-1859.

Russell, C. B., Stewart, R. C., and Dahlquist, F. W. (1989). Control of Transducer Methylation Levels in *Escherichia coli*: Investigation of Components Essential for Modulation of Methylation and Demethylation Reactions. J. Bacteriol. *171*, 3609-3618.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

San Millan, J. L., Boyd, D., Dalbey, R., Wickner, W., and Beckwith, J. (1989). Use of *pho*A Fusions to Study the Topology of the *Escherichia coli* Inner Membrane Protein Leader Peptidase. J. Bacteriol. *171*, 5536-5541.

Schleif, R. S. (1992). DNA Looping. Ann. Rev. Biochem. *61*, 199-223.