invitrogen™

pBAD Directional TOPO® Expression Kit

Five-minute, directional TOPO® cloning of blunt-end PCR products into vectors for soluble, regulated expression and purification in *E. coli*

Catalog no. K4202-01

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Manual part no. 25-0526

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User Manual

Contents

Kit Contents and Storage

Shipping/Storage

The pBAD Directional TOPO® Expression Kit is shipped on dry ice. Each kit contains a box with pBAD/D-TOPO® reagents (Box 1), a box with One Shot® TOP10 Chemically Competent *E. coli* (Box 2), and a stab of LMG194.
Store Box 1 at –20°C and Box 2 at –80°C. **Store the LMG194 stab at 4°C.**

pBAD/D-TOPO® Reagents

pBAD/D-TOPO® reagents (Box 1) are listed below. **Note that you must supply a thermostable, proofreading polymerase, and the appropriate PCR buffer. Store Box 1 at –20°C.**

Item	Concentration	Amount
pBAD202/D-TOPO® vector	15–20 ng/µL plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 µg/mL BSA 30 M bromophenol blue	20 µL
dNTP Mix	12.5 mM dATP; 12.5 mM dCTP 12.5 mM dGTP; 12.5 mM dTTP in water, pH 8	10 µL
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µL
Sterile Water	--	1 mL
20% L-Arabinose	20% in sterile water	1 mL
TrxFus Forward Sequencing Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL
pBAD Reverse Sequencing Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL
Control PCR Primers	0.1 µg/µL **each** in TE Buffer, pH 8	10 µL
Control PCR Template	0.1 µg/µL in TE Buffer, pH 8	10 µL
pBAD202/D/*lac*Z Expression Control Plasmid	0.01 µg/µL in TE buffer, pH 8	10 µL

Sequences of the Primers

The table below provides the sequences of the Trx Forward and pBAD Reverse sequencing primers. Two micrograms of each primer are supplied.

Primer	Sequence	pMoles Supplied
TrxFus Forward	5´-TTCCTCGACGCTAACCTG-3´	371
pBAD Reverse	5´-GATTTAATCTGTATCAGG-3´	363

Continued on next page

One Shot® TOP10 Reagents

The table below lists the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is 1×10^9 cfu/µg DNA. **Store Box 2 at –80°C.**

Item	Composition	Amount
TOP10 cells	--	21 × 50 µL
S.O.C. 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
pUC19 Control DNA	10 pg/µL in: 5 mM Tris-HCl 0.5 mM EDTA, pH 8.0	50 µL

Genotype of TOP10

Use this strain for general cloning of blunt-end PCR products into the pBAD202/D-TOPO® vector.

Genotype: 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

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 *ara*BADC. It is also streptomycin and tetracycline resistant.

Preparing LMG194 Glycerol Stocks

Store the LMG194 *E. coli* stab supplied with the kit at 4°C. Upon receipt, we recommend that you prepare a set of LMG194 glycerol master stocks within two weeks of receiving the kit.

1. Streak a small portion of the LMG194 cells from the stab on an LB plate containing the appropriate antibiotics and incubate at 37°C overnight.
2. Isolate a single colony and inoculate into 5–10 mL of LB medium with the appropriate antibiotics.
3. Grow the culture to stationary phase (OD_{600} = 1–2).
4. Mix 0.8 mL of culture with 0.2 mL of sterile glycerol and transfer to a cryovial.

Store at –80°C. Use one master stock to create working stocks for regular use.

Introduction

Description of the System

Product Features

The pBAD Directional TOPO® Expression Kit utilizes a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for soluble, regulated expression and simplified protein purification in *E. coli*. Blunt-end PCR products clone directionally at greater than 90% efficiency with no ligase, post-PCR procedures, or restriction enzymes required. In addition, pBAD202/D-TOPO® vector contains the His-Patch (HP) thioredoxin leader for increased translation efficiency and solubility of recombinant fusion proteins.

Expression in *E. coli* is driven by the *ara*BAD promoter (P_{BAD}). The AraC gene product encoded on the pBAD202/D-TOPO® vector positively regulates this promoter.

pBAD202/ D-TOPO® Vector

pBAD202/D-TOPO® is designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for regulated expression in *E. coli*. Features of the vector include:

- *ara*BAD promoter (P_{BAD}) for tight, dose-dependent regulation of heterologous gene expression
- N-terminal His-Patch thioredoxin for increased translation efficiency and solubility of heterologous proteins
- Directional TOPO® Cloning site for rapid and efficient directional cloning of a blunt-end PCR product (see next page for more information)
- C-terminal fusion tag for detection and purification of recombinant fusion proteins
- Kanamycin resistance gene for selection in *E. coli*
- *ara*C gene encoding a regulatory protein for tight regulation of the P_{BAD} promoter
- pUC origin for maintenance in *E. coli*.

  Note: Although the pBAD202/D-TOPO® vector contains a pUC origin, they act as low-copy number plasmids, resulting in lower yields of the vectors.

How Directional TOPO® Cloning Works

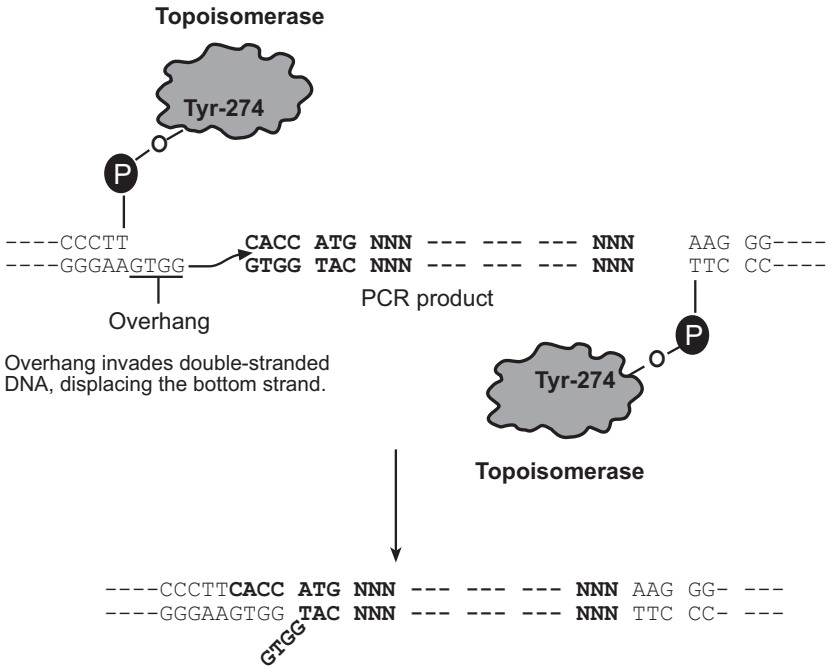
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



Arabinose Regulation and Thioredoxin

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 (0.1% to 0.2%). 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, see page 33 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 (see Holmgren, 1985 for a review). 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. When used as a fusion partner, thioredoxin can increase translation efficiency and, in some cases, solubility of eukaryotic proteins expressed in *E. coli*.

Examples of eukaryotic proteins that have been produced as soluble C-terminal fusions to the thioredoxin protein in *E. coli* (LaVallie *et al.*, 1993) include:

- Murine interleukin-2
- Human interleukin-3
- Murine interleukin-4
- Murine interleukin-5
- Human macrophage colony stimulating factor
- Murine steel factor
- Murine leukemia inhibitory factor
- Human bone morphogenetic protein-2

His-Patch Thioredoxin

The thioredoxin protein has been mutated to contain a metal binding domain, and is termed “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 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 been shown to have 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™).

Experiment Outline

Experiment Outline

The table below describes the general steps needed to clone and express your gene of interest. For more details, refer to the pages indicated.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the N-terminal His-Patch thioredoxin and C-terminal V5 epitope and polyhistidine tag, if desired. Consult the diagram on page 8 to help you design your PCR primers.	5–8
2	Produce your blunt-end PCR product.	9
3	TOPO® Clone your PCR product into pBAD202/D-TOPO® and transform into One Shot® TOP10 *E. coli*. Select transformants on LB plates containing the appropriate antibiotic.	10–14
4	Analyze transformants by restriction digestion or PCR.	15
5	Select a transformant with the correct restriction pattern and sequence it to confirm that your gene is cloned in frame with the N-terminal His-Patch thioredoxin and C-terminal V5 epitope and polyhistidine tag, if desired.	15
6	Select positive transformant and induce expression with arabinose.	17–18
7	Assay for expression of your protein of interest.	19–21

Methods

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Consider the following when designing your PCR primers:

- Sequences required to facilitate directional cloning (see below).
- Whether or not you wish to clone your PCR product in frame with the C-terminal V5 epitope and polyhistidine region.

Specific Features for Expression

The pBAD202/D-TOPO® vector contains the following features to facilitate expression:

- Initiation ATG that is properly spaced from the optimized ribosome binding site to ensure optimal translation.
- HP-thioredoxin, which acts as a translation leader to facilitate high-level expression and in some cases, solubility. HP-thioredoxin can be removed after protein purification using enterokinase (e.g. EKMax™, see page 34).

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to page 8 for a diagram of the TOPO® Cloning site for the pBAD202/D-TOPO® vector.

- To enable directional cloning, the forward PCR primer **must** contain the sequence CACC at the 5′ end of the primer (see **Example** below). The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in the pBAD202/D-TOPO® vector.
- If you wish to include the N-terminal thioredoxin, design the forward PCR primer to ensure that your protein is in frame with the N-terminal leader peptide.

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer:

DNA sequence: `5′-GTA GGA TCT GAT AAA`
Proposed Forward PCR primer: `5′-C ACC GTA GGA TCT GAT AAA`

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

Continued on next page

Designing PCR Primers, Continued

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the points below. Refer to page 8 for a diagram of the TOPO® Cloning site on the pBAD202/D-TOPO® vector.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 below).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- To fuse your PCR product in frame with the C-terminal V5 epitope tag and polyhistidine region, design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you do NOT wish to fuse your PCR product in frame with the C-terminal V5 epitope tag and polyhistidine region, include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag. The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

Continued on next page

Example #2 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. The stop codon is underlined.

…GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′

- To fuse the ORF in frame with a C-terminal tag, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.

- If you do not want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that the pBAD202/D-TOPO® vector accepts blunt-end PCR products. Refer to the diagram of the TOPO® Cloning site on page 8 to help you design your primers.
- When synthesizing PCR primers, **do not** add 5′ phosphates to the primers, because 5′ phosphates prevent the synthesized PCR product from ligating into the pBAD202/D-TOPO® vector.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

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Designing PCR Primers, Continued

TOPO® Cloning Site

Use the diagram below to design suitable PCR primers to clone and express your PCR product in pBAD202/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. **The vector sequence of pBAD202/D-TOPO® is available for downloading at www.invitrogen.com or by contacting Technical Support (page 36)**.

Producing Blunt-End PCR Products

Introduction

After you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. We recommend Platinum® *Pfx* DNA Polymerase, AccuPrime™ *Pfx* DNA Polymerase, or *Pfx50*™ DNA Polymerase, available separately from Invitrogen (see page 34 for ordering information). Follow the guidelines below to produce your blunt-end PCR product.

Materials Supplied by the User

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers for PCR product

Note: dNTPs (adjusted to pH 8) are provided in the kit.

Producing Blunt-End PCR Products

Set up a 25 µL or 50 µL PCR reaction using the guidelines below.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at –20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, gel-purify the desired product (see pages 26–27).
- Estimate the concentration of your PCR product. Use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Performing the TOPO® Cloning Reaction

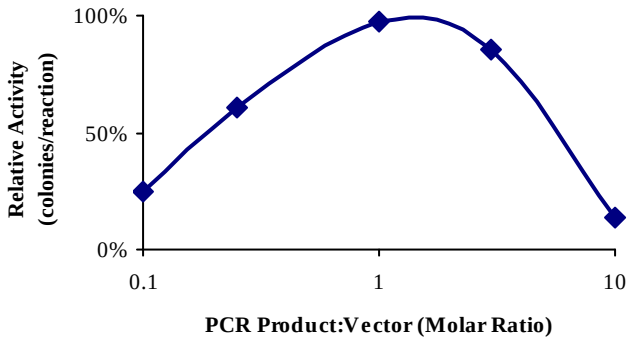
Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pBAD202/D-TOPO® vector and transform the recombinant vector into One Shot® TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure best results. We suggest that you read this section and the section entitled **Transforming One Shot® TOP10 Competent Cells** (pages 12–14) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 28–29 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1. These results are generally obtained if too little PCR product is used (i.e. PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For the pBAD202/D-TOPO® vector, using 1–5 ng of a 1 kb PCR product or 5–10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Performing the TOPO® Cloning Reaction, Continued

Using Salt Solution in the TOPO® Cloning Reaction

Perform TOPO® Cloning in a reaction buffer containing salt (i.e. using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page 34 for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, **reduce** the amount of salt in the TOPO® Cloning reaction to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.**

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µL	0.5 to 4 µL
Salt Solution	1 µL	--
Dilute Salt Solution (1:4)	--	1 µL
Sterile Water	add to a final volume of 5 µL	add to a final volume of 5 µL
TOPO® vector	1 µL	1 µL
Final volume	6 µL	6 µL

*Store all reagents at –20°C when finished. Store salt solutions and water at room temperature or 4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22–23°C).

   Note: For most applications, 5 minutes yields plenty of colonies for analysis. Depending on your needs, you can vary the length of the TOPO® Cloning reaction from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pBAD202/D-TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells. This section provides protocols to transform chemically competent or electrocompetent *E. coli*.

Materials Supplied by the User

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50 µg/mL kanamycin (two for each transformation)
- 37°C shaking and non-shaking incubator

Preparing for Transformation

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

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm LB plates containing 50 µg/mL kanamycin at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® TOP10 cells from Box 2 for each transformation.

Continued on next page

Transforming One Shot® TOP10 Competent Cells, Continued

Important

The number of colonies obtained after transforming the pBAD202/D-TOPO® vector into One Shot® TOP10 cells is generally much lower when compared to the number of colonies obtained after transforming other TOPO® vectors (e.g. pCR®T7 TOPO®).

- Directional TOPO® Cloning generally yields 2 to 5-fold fewer colonies than traditional bidirectional TOPO TA Cloning®.
- Transforming low-copy number TOPO® plasmids generally yields 2 to 5-fold fewer colonies than transforming high-copy number TOPO® plasmids.

To compensate for the lower transformation efficiency using the pBAD202/D-TOPO®:

- Increase the amount of TOPO® Cloning reaction that you transform into TOP10 cells (use 3 µL).
- Increase the amount of transformed cells that you plate (use 100–200 µL for chemically competent cells and 50–100 µL for electrocompetent cells).

Example: When directionally TOPO® Cloning a 750 bp test insert into the pBAD202/D-TOPO® vector, we generally obtain 500–1,500 total colonies. Although fewer total colonies are obtained, greater than 90% of the colonies contain plasmid with your PCR insert in the correct orientation.

One Shot® TOP10 Chemical Transformation Protocol

1. Add 3 µL of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µL of room temperature S.O.C. medium to the tubes.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 100–200 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Positive Clones**, page 15).

Continued on next page

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 3 µL of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a 0.1 cm cuvette containing 50 µL of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µL of room temperature S.O.C. medium to the cuvette.
4. Transfer the solution to a 15 mL snap-cap tube (e.g. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance gene.
5. Spread 50–100 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Positive Clones**, page 15).

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 µL and 80 µL (0.1 cm cuvettes) or 100 µL to 200 µL (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50 µg/mL kanamycin.
2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification or PureLink™ HiPure Plasmid Miniprep kits (see page 34 for ordering information). Refer to www.invitrogen.com or contact Technical Support for more information on a large selection of plasmid purification columns.

   Note: Because the pBAD202/D-TOPO® vector acts as a low-copy number plasmid, you may need to increase the amount of bacterial culture to obtain enough plasmid DNA for sequencing or analysis purposes. Use extra care during purification to obtain plasmid DNA of sufficiently pure quality for sequencing (see below).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is in frame with the N-terminal His-Patch thioredoxin peptide and the C-terminal V5 epitope and polyhistidine (6×His) tag. The TrxFus Forward and pBAD Reverse primers are included in the kit to help you sequence your insert. Refer to the diagram on page 8 for the location of the primer binding sites.

Important

If you download the sequence from www.invitrogen.com, note that the overhang sequence (GTGG) is shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Continued on next page

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the TrxFus Forward primer or the pBAD Reverse primer and a primer that hybridizes within your insert. Determine the amplification conditions based on the size of your insert and the sequence of your insert-specific primer. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are also suitable.

Materials Needed

PCR SuperMix High Fidelity (see page 34)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µL of PCR SuperMix High Fidelity into a 0.5 mL microcentrifuge tube. Add 1 µL each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µL of the PCR cocktail from Step 1, above.
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Analyze by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 28–29. These reactions will help you troubleshoot your experiment.

Long-Term Storage

After you have identified the correct clone, purify the colony and make a glycerol stock for long term storage. We recommend that you also store a stock of plasmid DNA at –20°C.

1. Streak the original colony out for single colony on LB plates containing 50 µL/mL kanamycin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µL/mL kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Expressing the PCR Product

Introduction

Because 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.

Using LMG194

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 P_{BAD} by glucose. **After you have determined that you have the correct construct, transform it into LMG194 prior to performing expression experiments.** Follow the guidelines below for using LMG194:

- Induce the pBAD promoter when cells are growing in LB or RM-Glucose.
- If you are growing your construct under maximal repression, i.e. with D-glucose in RM media, then you must spin down the culture and resuspend it in RM containing 0.2% glycerol and Arabinose (i.e. substitute glycerol for the glucose in the media recipe on page 25).

Plasmid Preparation

You may prepare plasmid DNA using any method. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit or the PureLink™ HiPure Plasmid Miniprep Kit (see page 34). Refer to www.invitrogen.com or contact Technical Support for more information on a large selection of plasmid purification columns.

Note that, because you are purifying a vector that acts as a low-copy number plasmid, you may need to increase the amount of bacterial culture that you use to prepare your plasmid construct.

Positive Control

pBAD202/D/*lac*Z is included in the kit as an expression control. This control plasmid contains directionally TOPO® Cloned gene encoding β-galactosidase (see page 32 for a map). Transform 10 ng of the control plasmid into One Shot® TOP10 cells using the procedure on page 13 or page 14.

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.** 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, try arabinose concentrations spanning the amount determined in Step 1, or perform a time course.

Note: If your protein is insoluble, analyze the supernatant **and** the pellet of lysed cells for expression of soluble protein (see page 19).

Continued on next page

Expressing the PCR Product, Continued

Materials Required

- SOB or LB containing 50 µg/mL kanamycin
- 37°C shaking incubator
- 20% L-arabinose (provided). Additional L-arabinose is available from Sigma (Cat. no. A3256).

Pilot Expression

In addition to testing your transformants, we recommend that you include pBAD202/D/*lac*Z 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.
   Note: If you are using LMG194 as a host, use RM medium containing glucose and 100 µg/mL ampicillin for overnight growth (see page 25 for a recipe), and then substitute glycerol for glucose in medium at Step 3 below (see **Using LMG194**, previous page).
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.
9. Use the stock solutions prepared in Step 6 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. You will have a total of ten samples for each transformant and two samples for each control. Proceed to **Analyzing Samples**, next page.

Analyzing Samples

Preparing Samples

Before starting, prepare SDS-PAGE gels or use one of the pre-cast polyacrylamide gels available from Invitrogen (see below) to analyze all the samples you have collected.

Note: To analyze your samples for soluble protein, see the next section.

1. When all the samples have been collected from Steps 8 and 11 on page 18, resuspend each cell pellet in 80 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 them at –20°C.

Preparing Samples for Soluble/Insoluble Protein

1. Thaw and resuspend each pellet in 500 µL of Lysis Buffer (see page 25 for recipe).
2. Freeze sample in dry ice or liquid nitrogen, and then thaw it at 42°C. Repeat 2 to 3 times.

   Note: To facilitate lysis, you may add lysozyme to the sample or sonicate the cells.
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-PAGE sample buffer and boil for 5 minutes.
5. Add 500 µL of 1X SDS-PAGE sample buffer to the 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 gel and electrophorese.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. The NuPAGE® Gel System avoids the protein modifications associated with LaemmLi-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to www.invitrogen.com or contact Technical Support (page 36).

Continued on next page

Analyzing Samples

To determine the success of your expression experiment, perform the following types of analyses:

1. Stain the polyacrylamide gel with Coomassie blue and look for a band of increasing intensity in the expected size range for the recombinant protein. Use the uninduced culture as a negative control.
2. Perform a western blot to confirm that the overexpressed band is your desired protein (see below); this is the preferred method for analyzing your samples.
3. Use the expression control plasmid to confirm that growth and induction were performed properly. The size of the β-galactosidase fusion protein expressed from the positive control plasmid when induced with 0.02% arabinose is approximately 133 kDa.
4. Determine the approximate arabinose concentration for maximum expression.

Detecting Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use antibodies against the appropriate epitope (see page 35 for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein is available from Invitrogen for use as a positive control for detection of fusion proteins containing a thioredoxin, V5, or C-terminal 6×His epitope. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to www.invitrogen.com or contact Technical Support (page 36).

Note

Expressing your protein with the N-terminal thioredoxin and/or the C-terminal peptide tags increases the size of your recombinant protein. The table below lists the expected increase in the molecular weight of your recombinant fusion protein. Be sure to account for any additional amino acids between the tag and your fusion protein.

Peptide Tag	Expected Size Increase (kDa)
N-terminal Thioredoxin	13 kDa
C-terminal V5, 6×His	3 kDa

Continued on next page

Optimizing Expression

After you have detected expression of your protein of interest, perform some experiments to further optimize expression.

- Use the **Pilot Expression** protocol on page 18, but vary the arabinose concentration over a smaller range. For example, if you obtained the best expression at 0.002% arabinose in the medium, try 0.0004%, 0.0008%, 0.001%, 0.004%, and 0.008%.
- You may also perform a time course of induction to determine if varying the time increases expression. Take time points every hour, over a 5 to 6 hour period.
- If your protein is insoluble, analyze the supernatant and pellet of lysed cells when you vary the arabinose concentration (see **Preparing Samples for Soluble/Insoluble Protein**, page 19).
- Store your cell lysates at –20°C.

Expressing Toxic Proteins

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

1. Transform your construct into LMG194. LMG194 can be grown in RM medium that enables repression of *ara*BAD promoter by glucose or glycerol (see **Using LMG194**, page 17).
2. Follow the Pilot Expression protocol (page 18) using RM medium containing 0.2% glucose or glycerol (see page 25 for recipe) to grow the cells.
3. Monitor the OD_{600}, because the cells grow more slowly in RM medium.
4. Induce with various concentrations of arabinose as described in the Pilot Expression protocol.
5. Monitor OD_{600} over time to make sure that the cells are growing.

Purifying Recombinant Fusion Proteins

You may use the ProBond™ Purification System, the Ni-NTA Purification System, or a similar product to purify your 6×His-tagged protein (see page 35 for ordering information). Both purification systems contain a metal-chelating resin specifically designed to purify 6×His-tagged proteins. Before starting, consult the ProBond™ or Ni-NTA Purification System manual to familiarize yourself with the buffers and the binding and elution conditions. If you are using another resin, follow the manufacturer's instructions.

Removing the N-terminal Leader by Enterokinase

The enterokinase (EK) recognition site can be used to remove the N-terminal leader from your recombinant fusion protein after purification. Note that after digestion with enterokinase, there will be seven vector-encoded amino acids remaining at the N-terminus of the protein (see page 8).

A recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) is available from Invitrogen. Instructions for digestion are included with the product. To remove EKMax™ from the digest, you may use EK-Away™ Resin, also available from Invitrogen (see page 34 for ordering information).

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions in parallel with your samples (see pages 28–29).

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR product too dilute	• Concentrate the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Long PCR product	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (i.e. does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.

Continued on next page

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence CACC at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Large number of incorrect inserts cloned	PCR reaction contains artifacts (i.e. does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at –80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	One Shot® transformation protocol not followed correctly	Follow the One Shot® transformation protocol provided on page 13 or page 14.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

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. 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.
3. After autoclaving, cool to ~55°C, add antibiotic if needed, and pour into 10 cm plates.
4. Let the plates harden, then invert them, and store at 4°C, in the dark.

SOB Medium

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 antibiotic, if needed.
5. Store the medium at 4°C. **Medium is stable for only 1–2 weeks.**

Continued on next page

RM Medium + Glucose

1X M9 Salts (see below for recipe for 10X M9 Salts)
2% Casamino Acids
0.2% glucose
1 mM $MgCl_2$
antibiotic to the appropriate concentration

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
antibiotic	

4. Mix well and store medium containing antibiotic 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 the solution 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 the buffer at 4°C.

Purifying the PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to Current Protocols in Molecular Biology, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Two simple protocols are described below.

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit allows you to rapidly purify PCR products from regular agarose gels (see page 34 for ordering information).

1. Equilibrate a water bath or heat block to 50°C.
2. Cut the area of the gel containing the desired DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment. Weigh the gel slice.
3. Add Gel Solubilization Buffer (GS1) supplied in the kit as follows:
   - For ≤2% agarose gels, place up to 400 mg gel into a sterile, 1.5-mL polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µL Gel Solubilization Buffer (GS1) for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5-mL polypropylene tubes and add 60 µL Gel Solubilization Buffer (GS1) for every 10 mg of gel.
4. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After gel slice appears dissolved, incubate for an **additional** 5 minutes.
5. Preheat an aliquot of TE Buffer (TE) to 65–70°C
6. Place a Quick Gel Extraction Column into a Wash Tube. Pipette the mixture from Step 4, above onto the column. Use 1 column per 400 mg agarose.
7. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
8. **Optional:** Add 500 µL Gel Solubilization Buffer (GS1) to the column. Incubate at room temperature for 1 minute. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
9. Add 700 µL Wash Buffer (W9) with ethanol (add 96–100% ethanol to the Wash Buffer according to instructions on the label of the bottle) to the column and incubate at room temperature for 5 minutes. Centrifuge at >12,000 × g for 1 minute. Discard flow-through.
10. Centrifuge the column at >12,000 × g for 1 minute to remove any residual buffer. Place the column into a 1.5 mL Recovery Tube.
11. Add 50 µL **warm** (65–70°C) TE Buffer (TE) to the center of the cartridge. Incubate at room temperature for 1 minute.
12. Centrifuge at >12,000 × g for 2 minutes. **The Recovery Tube contains the purified DNA.** Store DNA at –20°C. Discard the column.
13. Use 4 µL of the purified DNA for the TOPO® Cloning reaction.

Continued on next page

Low-Melt Agarose Method

Note that gel purification will dilute your PCR product. Use only chemically competent cells for transformation.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Use 4 µL of the melted agarose containing your PCR product in the TOPO® Cloning reaction (page 10).
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes** to keep the agarose melted.
7. Transform 2 to 4 µL directly into TOP10 One Shot® cells using the method on page 13.

Note

Cloning efficiency may decrease with purification of the PCR product. To produce a single band, optimize your PCR conditions.

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/mL kanamycin (see page 24 for recipe).

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µL PCR:

Control DNA Template (10 ng)	1 µL
10X PCR Buffer (appropriate for enzyme)	5 µL
dNTP Mix	0.5 µL
Control PCR Primers (0.1 µg/µL)	1 µL
Sterile Water	41.5 µL
Thermostable polymerase (1–2.5 unit/µL)	1 µL
Total Volume	50 µL

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible.

4. Estimate the concentration of the PCR product, and adjust the concentration as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (i.e. 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

Continued on next page

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pBAD/D-TOPO® vector, set up two 6 µL TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µL	3 µL
Salt Solution	1 µL	1 µL
Control PCR Product	--	1 µL
pBAD/D-TOPO® vector	1 µL	1 µL
Final volume	6 µL	6 µL

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 3 µL of each reaction into separate vials of One Shot® TOP10 competent cells (page 13 or 14).
4. Spread 100–200 µL of each transformation mix onto LB plates containing 50 µg/mL kanamycin. Plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Analyzing Results

The vector + PCR insert reaction should yield hundreds of colonies. To analyze the transformations, isolate plasmid DNA, and digest with *Hin*d III or another appropriate restriction enzyme. The table below lists the *Hin*d III digestion pattern that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Pattern (bp)
pBAD202/D-TOPO®	*Hin*d III	Correct orientation: 560, 4642 Reverse orientation: 226, 4976 Empty vector: 4448

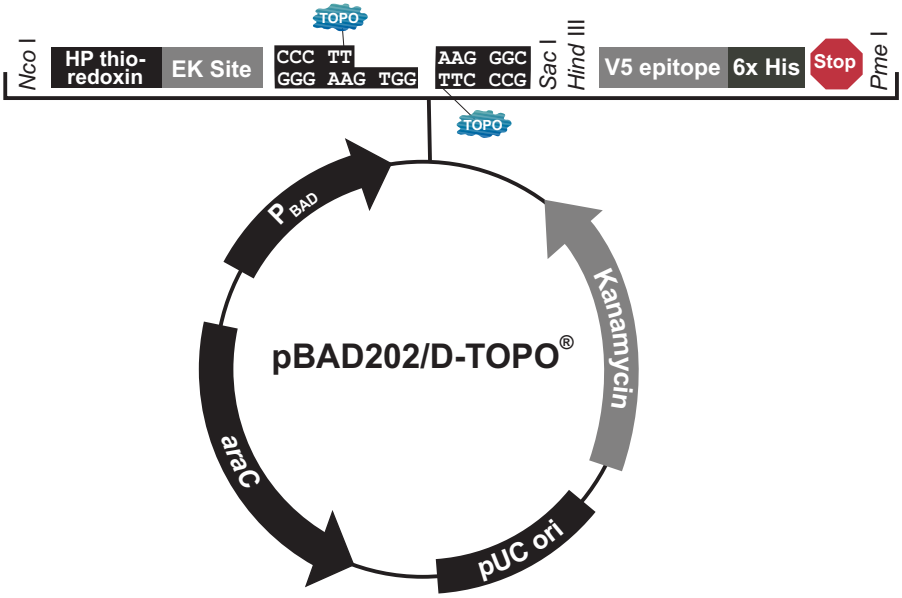
Transformation Control

pUC19 plasmid is included as a control to check the transformation efficiency of One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 13 or 14. Plate 10 µL of the transformation mixture plus 20 µL of S.O.C. on LB plates containing 100 µg/mL ampicillin. Transformation efficiency should be ~1×10^9 cfu/µg DNA.

Map and Features of pBAD202/D-TOPO®

Map of pBAD202/ D-TOPO®

The map below shows the features of pBAD202/D-TOPO®. **The complete sequence of the vector is available for downloading at www.invitrogen.com or from Technical Support (page 36).**



pBAD202/D-TOPO®
4448 nucleotides

Arabinose O_2 operator region:	4-19
Arabinose O_1 operator region:	161-182
CAP binding site:	203-216
Arabinose I_1 and I_2 region:	213-251
Arabinose minimal promoter:	248-276
Ribosome binding site:	329-332
His-Patch Thioredoxin ORF:	346-674
TrxFus Forward priming site:	655-672
Enterokinase recognition site:	691-705
TOPO® recognition site 1:	718-722
Overhang:	723-726
TOPO® recognition site 2:	727-731
V5 epitope:	748-789
Polyhistidine (6xHis) region:	799-816
pBAD Reverse priming site:	872-889
*rrn*B transcriptional termination region:	922-1079
Kanamycin resistance gene (c):	1448-2242
pUC origin:	2340-3013
araC ORF (c):	3544-4422
(c) = complementary strand	

Continued on next page

Map and Features of pBAD202/D-TOPO®, Continued

Features of pBAD202/D-TOPO®

The pBAD202/D-TOPO® vector contains the following elements. 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).
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).
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}.
Ribosome binding site	Increases efficiency of recombinant fusion protein expression.
HP-thioredoxin	Provides a highly efficient fusion partner for translation of the fusion protein.
TrxFus forward priming site	Allows sequencing of the insert in the sense orientation.
Enterokinase recognition site (Asp-Asp-Asp-Asp-Lys)	Allows removal of the N-terminal tag from the recombinant fusion protein using an enterokinase such as EKMax™.
TOPO® Cloning site (directional)	Allows rapid cloning of your PCR product for expression in *E. coli.*
C-terminal V5 epitope tag (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991).
C-terminal 6×His tag	Allows purification of the recombinant fusion protein on metal-chelating resins (e.g. ProBond™). Allows detection of the recombinant fusion protein with the Anti-His(C-term) Antibodies (Lindner *et al.*, 1997).
*rrn*B transcription termination region	Strong transcription termination region.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin	Allows 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).

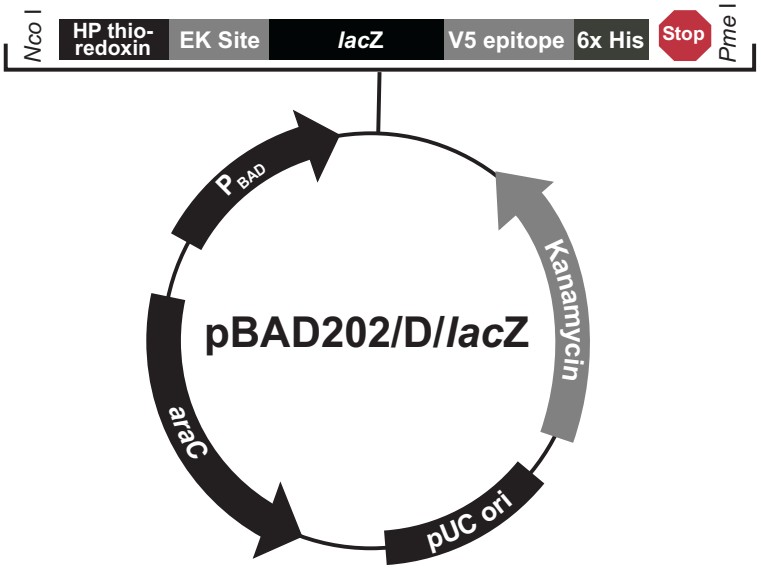
Map of pBAD202/D/*lac*Z

Description

pBAD202/D/*lac*Z (7,520 bp) is a control vector that contains the gene for β-galactosidase. The *lac*Z gene was amplified and directionally TOPO® Cloned into pBAD202/D-TOPO® in frame with HP-thioredoxin and the C-terminal peptide containing the V5 epitope and a polyhistidine (6×His) tag. The size of the β-galactosidase fusion protein is approximately 133 kDa.

Map of pBAD202/D/*lac*Z

The figure below shows the elements of pBAD202/D/*lac*Z. **The complete sequence of the vector is available at www.invitrogen.com or by contacting Technical Support (page 36).**



pBAD202/D/*lac*Z
7,520 nucleotides

Arabinose O_2 operator region:	4-19
Arabinose O_1 operator region:	161-182
CAP binding site:	203-216
Arabinose I_1 and I_2 region:	213-251
Arabinose minimal promoter:	248-276
Ribosome binding site:	329-332
His-Patch Thioredoxin ORF:	346-674
TrxFus Forward priming site:	655-672
Enterokinase recognition site:	691-705
*lac*Z ORF:	730-3801
V5 epitope:	3820-3861
Polyhistidine (6xHis) region:	3871-3888
pBAD Reverse priming site:	3944-3961
*rrn*B transcriptional termination region:	3994-4151
Kanamycin resistance gene (c):	4520-5314
pUC origin:	5412-6085
araC ORF (c):	6616-7494

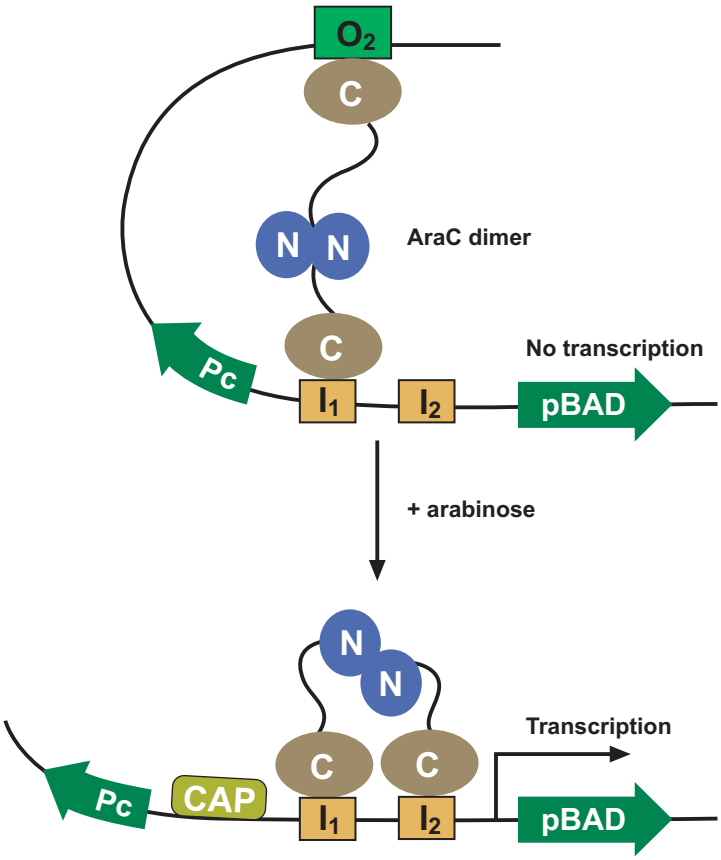
(c) = complementary strand

Regulation by Arabinose

Regulation of the P_{BAD} Promoter

The *ara*BAD promoter used in the pBAD/D-TOPO® vectors 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.

Accessory Products

Additional Products

Many of the reagents supplied with the pBAD Directional TOPO® Expression Kit and other reagents suitable for use with the kit are available separately from Invitrogen. Ordering information for these reagents is provided below. For details, visit www.invitrogen.com.

Product	Amount	Cat. no.
PCR Optimizer™ Kit	100 reactions	K1220-01
PCR SuperMix High Fidelity	100 reactions	10790-020
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink™ HiPure Plasmid Miniprep Kit	25 preps	K2100-02
	100 preps	K2100-03
EKMax™ Enterokinase	250 units	E180-01
EK-Away™ Resin	7.5 mL	R180-01
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
β-Gal Antiserum	50 µL	R901-25
β-Gal Assay Kit	100 reactions	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
X-gal	100 mg	15520-034

Proofreading DNA Polymerases

Invitrogen offers a variety of proofreading, thermostable DNA polymerases for generating blunt-end PCR products. Ordering information is provided below.

Item	Amount	Cat. no.
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
AccuPrime™ *Pfx* DNA Polymerase	200 reactions	12344-024
Pfx50™ DNA Polymerase	100 reactions	12355-012

Electrocompetent Cells

TOP10 cells are also available as electrocompetent cells. See the table below for ordering information.

Kit	Reactions	Cat. no.
One Shot® TOP10 Electrocomp™ *E. coli*	10	C4040-50
	20	C4040-52
TOP10 Electrocomp™ Kits	20	C664-55
	40	C664-11
	120	C664-24

Continued on next page

Detecting Recombinant Protein

Expression of your recombinant fusion protein can be detected using Anti-Thio, Anti-V5, or Anti-His(C-term) antibodies available from Invitrogen. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemiluminescent or colorimetric detection methods. The amount of antibody supplied is sufficient for 25 Western blots.

Product	Epitope	Cat. no.
Anti-Thio™ Antibody	Detects His-Patch thioredoxin fusion proteins. **Note:** The exact epitope detected by this antibody has not been mapped.	R920-25
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991). GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His(C-term) Antibody	Detects the C-terminal polyhistidine (6×His) tag, requires the free carboxyl group for detection (Lindner *et al.*, 1997). HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25

Purifying Recombinant Protein

If your gene of interest in is frame with the C-terminal peptide containing the polyhistidine (6×His) tag, you may use Invitrogen's ProBond™ or Ni-NTA Purification System to purify your recombinant fusion protein. See the table below for ordering information.

Product	Amount	Cat. no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Chelating Resin	50 mL	R801-01
	150 mL	R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 mL	R901-01
	25 mL	R901-15
Purification Columns (10 mL polypropylene columns)	50 columns	R640-50

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

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

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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MSDS

MSDSs (Material Safety Data Sheets) are available at www.invitrogen.com/msds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product and is searchable by product lot number, which is printed on each box. CofAs are .available on our website at www.invitrogen.com/support.

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Introduction

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Purchaser Notification, Continued

Limited Use Label License No. 29: ThioFusion™

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Information for European Customers

The LMG194 cell line is genetically modified. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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