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

Revision date: 7 June 2010
Manual part no. 25-0526
MAN000286
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.

<table>
<thead>
<tr>
<th>Item</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD202/D-TOPO® vector</td>
<td>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</td>
<td>20 μL</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>12.5 mM dATP; 12.5 mM dCTP; 12.5 mM dGTP; 12.5 mM dTTP in water, pH 8</td>
<td>10 μL</td>
</tr>
<tr>
<td>Salt Solution</td>
<td>1.2 M NaCl, 0.06 M MgCl₂</td>
<td>50 μL</td>
</tr>
<tr>
<td>Sterile Water</td>
<td></td>
<td>1 mL</td>
</tr>
<tr>
<td>20% L-Arabinose</td>
<td>20% in sterile water</td>
<td>1 mL</td>
</tr>
<tr>
<td>TrxFus Forward Sequencing Primer</td>
<td>0.1 μg/μL in TE Buffer, pH 8</td>
<td>20 μL</td>
</tr>
<tr>
<td>pBAD Reverse Sequencing Primer</td>
<td>0.1 μg/μL in TE Buffer, pH 8</td>
<td>20 μL</td>
</tr>
<tr>
<td>Control PCR Primers</td>
<td>0.1 μg/μL each in TE Buffer, pH 8</td>
<td>10 μL</td>
</tr>
<tr>
<td>Control PCR Template</td>
<td>0.1 μg/μL in TE Buffer, pH 8</td>
<td>10 μL</td>
</tr>
<tr>
<td>pBAD202/D/lacZ Expression Control Plasmid</td>
<td>0.01 μg/μL in TE buffer, pH 8</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>pMoles Supplied</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrxFus Forward</td>
<td>5´-TTCCCTCGACGCTAACCCTG-3´</td>
<td>371</td>
</tr>
<tr>
<td>pBAD Reverse</td>
<td>5´-GATTTAATCTGTATCAGG-3´</td>
<td>363</td>
</tr>
</tbody>
</table>

Continued on next page
Kit Contents and Storage, Continued

**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 $1 \times 10^8$ cfu/μg DNA. Store Box 2 at –80°C.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOP10 cells</td>
<td>--</td>
<td>$21 \times 50 \mu L$</td>
</tr>
<tr>
<td>S.O.C. Medium</td>
<td>2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl₂ 10 mM MgSO₄ 20 mM glucose</td>
<td>6 mL</td>
</tr>
<tr>
<td>pUC19 Control DNA</td>
<td>10 pg/μL in: 5 mM Tris-HCl 0.5 mM EDTA, pH 8.0</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

**Genotype of TOP10**

Use this strain for general cloning of blunt-end PCR products into the pBAD202/D-TOP10® vector.

**Genotype:** F– mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG

**Genotype of LMG194**

F– ΔlacX74 galE thi rpsL ΔphoA (Pvu II) Δara714 leu::Tn10.

**Note:** This strain is deleted for araBADC. 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 (OD₆₀₀ = 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 *araBAD* 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:

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>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.</td>
<td>5–8</td>
</tr>
<tr>
<td>2</td>
<td>Produce your blunt-end PCR product.</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>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.</td>
<td>10–14</td>
</tr>
<tr>
<td>4</td>
<td>Analyze transformants by restriction digestion or PCR.</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>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.</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Select positive transformant and induce expression with arabinose.</td>
<td>17–18</td>
</tr>
<tr>
<td>7</td>
<td>Assay for expression of your protein of interest.</td>
<td>19–21</td>
</tr>
</tbody>
</table>
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

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

Continued on next page
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).

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.
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

- 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).

Continued on next page
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).

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**O2 Region**

1  AAGAAACAAA TTGTCGATAT TGCAATACCA ATTCGCGTCA CTGTTCCTTT TACTGCTTAC CAAACGCGTA

81  ACCCGCTTTA TTAAGGACAT TGTAATACCA AGCGGACCCA AGACCATGAC AAAAAACGGT AAAAAAGTT TTCTAAATCA

161  GGGCAGAAAA GTCACTATTG ACTTATGTCG CGCGTCAAC CTTGCTATGC CGACATATT TTATACCATA AGATTACGGG

241  ATCTAAGCTT AAGCTTTTTA TGCACTTTTC TCCATACCCT TCTATTTTGGG CATGAAATAA TTTGTTTAA

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**O1 Region**

1  AAGAAACAAA TTGTCGATAT TGCAATACCA ATTCGCGTCA CTGTTCCTTT TACTGCTTAC CAAACGCGTA

81  ACCCGCTTTA TTAAGGACAT TGTAATACCA AGCGGACCCA AGACCATGAC AAAAAACGGT AAAAAAGTT TTCTAAATCA

161  GGGCAGAAAA GTCACTATTG ACTTATGTCG CGCGTCAAC CTTGCTATGC CGACATATT TTATACCATA AGATTACGGG

241  ATCTAAGCTT AAGCTTTTTA TGCACTTTTC TCCATACCCT TCTATTTTGGG CATGAAATAA TTTGTTTAA

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**RBS**

321  CTTAAGAAA GAGATATACA TACG ATG GGA TCT GAT AAA ATT ATT CAT CTG ACT GAT GAT TCT TTT GAT

391  ACT GAT GTA CTT AAG GCA GAT GGT GCA ATC CTG GTT GAT TTT TAC GCA TTC TGG GCA CAC TGG TGC

457  AAA ATG ATC GCT CCG ATT CTG GAT GAA ATC GCT GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA

523  CTG AAC ATC GAT CAC AAC CCG GCC ACT GCG CCG AAA TAT GGC ATC CTG GGT ATC CGG ACT CTG CTG

589  CTG TAC AAA AAC GGT GAA GTG GCC GCA ACC AAA GTG GTG GCA CTG TCT AAA GTG CAG TGG AAA AGG

655  TAC TTC AAA AAC GGT GAA GTG GCC GCA ACC AAA GTG GTG GCA CTG TCT AAA GTG CAG TGG AAA AGG

721  TAC ACC AAA AAC GGT GAA GTG GCC GCA ACC AAA GTG GTG GCA CTG TCT AAA GTG CAG TGG AAA AGG

781  GAT TCT AC GGT ACC GCT CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT CAT

850  GCCGATGAGA GAGAGTTTTC AGGCTTGATAG AGGATTAAATC AGAACCGAGA AGCGGTCGTA TAAAGACGAA TTTGCGCTGCG

930  GCCGATGAGA GAGAGTTTTC AGGCTTGATAG AGGATTAAATC AGAACCGAGA AGCGGTCGTA TAAAGACGAA TTTGCGCTGCG

1010  TCCCCAGAGC AGAGATGAGA AAGTAATATC ATCCGCGGTC ACAAATAAA ACGAAACGCT CGTCCGAGAAG ACTGGCGCTTA TCCCGCTTATC

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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.

![Graph showing TOPO® Cloning efficiency vs. PCR Product:Vector ratio]

Continued on next page
Perform TOPO\textsuperscript® 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\textsuperscript® 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 \textit{E. coli}, use the stock Salt Solution as supplied and set up the TOPO\textsuperscript® Cloning reaction as directed below.
- If you are transforming electrocompetent \textit{E. coli}, reduce the amount of salt in the TOPO\textsuperscript® Cloning reaction to 50 mM NaCl, 2.5 mM MgCl\textsubscript{2} to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM MgCl\textsubscript{2} Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO\textsuperscript® Cloning reaction as directed below.

### Using Salt Solution in the TOPO\textsuperscript® Cloning Reaction

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

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

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Chemically Competent \textit{E. coli}</th>
<th>Electrocompetent \textit{E. coli}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product</td>
<td>0.5 to 4 μL</td>
<td>0.5 to 4 μL</td>
</tr>
<tr>
<td>Salt Solution</td>
<td>1 μL</td>
<td>--</td>
</tr>
<tr>
<td>Dilute Salt Solution (1:4)</td>
<td>--</td>
<td>1 μL</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>add to a final volume of 5 μL</td>
<td>add to a final volume of 5 μL</td>
</tr>
<tr>
<td>TOPO\textsuperscript® vector</td>
<td>1 μL</td>
<td>1 μL</td>
</tr>
<tr>
<td>Final volume</td>
<td>6 μL</td>
<td>6 μL</td>
</tr>
</tbody>
</table>

*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\textsuperscript® 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\textsuperscript® 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\textsuperscript® TOP10 Competent Cells, next page.

**Note:** You may store the TOPO\textsuperscript® 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
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*
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, Continued

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 PBAD 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/lacZ 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/lacZ 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.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Stock Solution</th>
<th>Volume (mL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.002%</td>
<td>0.09</td>
<td>0.00002%</td>
</tr>
<tr>
<td>2</td>
<td>0.02%</td>
<td>0.09</td>
<td>0.0002%</td>
</tr>
<tr>
<td>3</td>
<td>0.2%</td>
<td>0.09</td>
<td>0.002%</td>
</tr>
<tr>
<td>4</td>
<td>2%</td>
<td>0.09</td>
<td>0.02%</td>
</tr>
<tr>
<td>5</td>
<td>20%</td>
<td>0.09</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

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
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.

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).

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.

<table>
<thead>
<tr>
<th>Peptide Tag</th>
<th>Expected Size Increase (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal Thioredoxin</td>
<td>13 kDa</td>
</tr>
<tr>
<td>C-terminal V5, 6×His</td>
<td>3 kDa</td>
</tr>
</tbody>
</table>

Continued on next page
Analyzing Samples, Continued

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 araBAD 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 araBAD 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 OD600 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 OD600 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).

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Few or no colonies obtained from sample reaction and the transformation control gave colonies</td>
<td>Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction</td>
<td>Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.</td>
</tr>
<tr>
<td>Too much PCR product used in the TOPO® Cloning reaction</td>
<td></td>
<td>Dilute the PCR product. Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.</td>
</tr>
<tr>
<td>PCR product too dilute</td>
<td></td>
<td>Concentrate the PCR product. Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.</td>
</tr>
<tr>
<td>PCR primers contain 5’ phosphates</td>
<td></td>
<td>Do not add 5’ phosphates to your PCR primers.</td>
</tr>
<tr>
<td>Incorrect PCR primer design</td>
<td></td>
<td>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.</td>
</tr>
<tr>
<td>Used <em>Taq</em> polymerase or a <em>Taq</em>/proofreading polymerase mixture for PCR</td>
<td></td>
<td>Use a proofreading polymerase for PCR.</td>
</tr>
<tr>
<td>Long PCR product</td>
<td></td>
<td>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.</td>
</tr>
<tr>
<td>PCR reaction contains artifacts (i.e. does not run as a single, discrete band on an agarose gel)</td>
<td></td>
<td>Optimize your PCR using the proofreading polymerase of choice. Gel-purify your PCR product to remove primer-dimers and smaller PCR products.</td>
</tr>
<tr>
<td>Cloning large pool of PCR products or a toxic gene</td>
<td></td>
<td>Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.</td>
</tr>
</tbody>
</table>
### Troubleshooting, Continued

**TOPO® Cloning Reaction and Transformation, continued**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorrect PCR primer design</td>
<td>Make sure that the forward PCR primer contains the sequence CACC at the 5’ end.</td>
<td></td>
</tr>
<tr>
<td>Reverse PCR primer is complementary to the GTGG overhang at the 5’ end</td>
<td>Make sure that the reverse PCR primer <strong>does not</strong> contain the sequence CACC at the 5’ end.</td>
<td></td>
</tr>
<tr>
<td>Large percentage of inserts cloned in the incorrect orientation</td>
<td>Make sure that the reverse PCR primer <strong>does not</strong> contain the sequence CACC at the 5’ end.</td>
<td></td>
</tr>
</tbody>
</table>
| 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 MgCl₂

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 MgCl₂. 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*
Recipes, Continued

**RM Medium + Glucose**

1X M9 Salts (see below for recipe for 10X M9 Salts)
2% Casamino Acids
0.2% glucose
1 mM MgCl₂
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 MgCl₂: 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:
- Na₂HPO₄: 60 g
- KH₂PO₄: 30 g
- NaCl: 5 g
- NH₄Cl: 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 KH₂PO₄ and K₂HPO₄.
2. For 100 mL, dissolve the following reagents in 90 mL of deionized water:
   - 0.3 mL KH₂PO₄
   - 4.7 mL K₂HPO₄
   - 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
Purifying the PCR Products, Continued

**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:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>2 minutes</td>
<td>94°C</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>1 minute</td>
<td>94°C</td>
<td>25X</td>
</tr>
<tr>
<td>Annealing</td>
<td>1 minute</td>
<td>55°C</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>1 minute</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>7 minutes</td>
<td>72°C</td>
<td>1X</td>
</tr>
</tbody>
</table>

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
Performing the Control Reactions, Continued

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:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>&quot;Vector Only&quot;</th>
<th>&quot;Vector + PCR Insert&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Water</td>
<td>4 μL</td>
<td>3 μL</td>
</tr>
<tr>
<td>Salt Solution</td>
<td>1 μL</td>
<td>1 μL</td>
</tr>
<tr>
<td>Control PCR Product</td>
<td>--</td>
<td>1 μL</td>
</tr>
<tr>
<td>pBAD/D-TOPO® vector</td>
<td>1 μL</td>
<td>1 μL</td>
</tr>
<tr>
<td>Final volume</td>
<td>6 μL</td>
<td>6 μL</td>
</tr>
</tbody>
</table>

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 Hind III or another appropriate restriction enzyme. The table below lists the Hind III digestion pattern that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Restriction Enzyme</th>
<th>Expected Digestion Pattern (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD202/D-TOPO®</td>
<td>Hind III</td>
<td>Correct orientation: 560, 4642</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse orientation: 226, 4976</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Empty vector: 4448</td>
</tr>
</tbody>
</table>

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 \( \sim 1 \times 10^9 \) cfu/μg DNA.
Map and Features 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).

---

[Map of pBAD202/D-TOPO®]

---

<table>
<thead>
<tr>
<th>Feature</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose O₂ operator region</td>
<td>4-19</td>
</tr>
<tr>
<td>Arabinose O₁ operator region</td>
<td>161-182</td>
</tr>
<tr>
<td>CAP binding site</td>
<td>203-216</td>
</tr>
<tr>
<td>Arabinose I₁ and I₂ region</td>
<td>213-251</td>
</tr>
<tr>
<td>Arabinose minimal promoter</td>
<td>248-276</td>
</tr>
<tr>
<td>Ribosome binding site</td>
<td>329-332</td>
</tr>
<tr>
<td>His-Patch Thioredoxin ORF</td>
<td>346-674</td>
</tr>
<tr>
<td>TrxFus Forward priming site</td>
<td>655-672</td>
</tr>
<tr>
<td>Enterokinase recognition site</td>
<td>691-705</td>
</tr>
<tr>
<td>TOPO® recognition site 1</td>
<td>718-722</td>
</tr>
<tr>
<td>Overhang</td>
<td>723-726</td>
</tr>
<tr>
<td>TOPO® recognition site 2</td>
<td>727-731</td>
</tr>
<tr>
<td>V5 epitope</td>
<td>748-789</td>
</tr>
<tr>
<td>Polhistidine (6xHis) region</td>
<td>799-816</td>
</tr>
<tr>
<td>pBAD Reverse priming site</td>
<td>872-889</td>
</tr>
<tr>
<td>rmB transcriptional termination region</td>
<td>922-1079</td>
</tr>
<tr>
<td>Kanamycin resistance gene (c)</td>
<td>1448-2242</td>
</tr>
<tr>
<td>pUC origin</td>
<td>2340-3013</td>
</tr>
<tr>
<td>araC ORF (c)</td>
<td>3544-4422</td>
</tr>
<tr>
<td>(c) = complementary strand</td>
<td></td>
</tr>
</tbody>
</table>

Continued on next page
The pBAD202/D-TOPO® vector contains the following elements. All features have been functionally tested.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ara</em>BAD promoter (<em>P</em>&lt;sub&gt;BAD&lt;/sub&gt;)</td>
<td>Provides tight, dose-dependent regulation of heterologous gene expression (Guzman <em>et al.</em>, 1995).</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt; region</td>
<td>Binding site of AraC that represses transcription from <em>P</em>&lt;sub&gt;BAD&lt;/sub&gt;.</td>
</tr>
<tr>
<td>O&lt;sub&gt;1&lt;/sub&gt; region</td>
<td>Binding site of AraC that represses transcription of the <em>araC</em> promoter (<em>P</em>&lt;sub&gt;C&lt;/sub&gt;) (transcribed on the opposite strand).</td>
</tr>
<tr>
<td>CAP binding site</td>
<td>Site where CAP (cAMP binding protein) binds to activate transcription from <em>P</em>&lt;sub&gt;BAD&lt;/sub&gt; and <em>P</em>&lt;sub&gt;C&lt;/sub&gt;.</td>
</tr>
<tr>
<td>I&lt;sub&gt;2&lt;/sub&gt; and I&lt;sub&gt;1&lt;/sub&gt; regions</td>
<td>Binding sites of AraC that activate transcription from <em>P</em>&lt;sub&gt;BAD&lt;/sub&gt;.</td>
</tr>
<tr>
<td>−10 and −35 regions</td>
<td>Binding sites of RNA polymerase for transcription from <em>P</em>&lt;sub&gt;BAD&lt;/sub&gt;.</td>
</tr>
<tr>
<td>Ribosome binding site</td>
<td>Increases efficiency of recombinant fusion protein expression.</td>
</tr>
<tr>
<td>HP-thioredoxin</td>
<td>Provides a highly efficient fusion partner for translation of the fusion protein.</td>
</tr>
<tr>
<td>TrxFus forward priming site</td>
<td>Allows sequencing of the insert in the sense orientation.</td>
</tr>
<tr>
<td>Enterokinase recognition site (Asp-Asp-Asp-Asp-Lys)</td>
<td>Allows removal of the N-terminal tag from the recombinant fusion protein using an enterokinase such as EKMax™.</td>
</tr>
<tr>
<td>TOPO® Cloning site (directional)</td>
<td>Allows rapid cloning of your PCR product for expression in <em>E. coli</em>.</td>
</tr>
<tr>
<td>C-terminal V5 epitope tag (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)</td>
<td>Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies (Southern <em>et al.</em>, 1991).</td>
</tr>
<tr>
<td>C-terminal 6×His tag</td>
<td>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 <em>et al.</em>, 1997).</td>
</tr>
<tr>
<td><em>rrnB</em> transcription termination region</td>
<td>Strong transcription termination region.</td>
</tr>
<tr>
<td>Kanamycin resistance gene</td>
<td>Allows selection of the plasmid in <em>E. coli</em>.</td>
</tr>
<tr>
<td>pUC origin</td>
<td>Allows low-copy replication and growth in <em>E. coli</em>.</td>
</tr>
<tr>
<td><em>araC</em> gene</td>
<td>Encodes the regulatory protein for tight regulation of the <em>P</em>&lt;sub&gt;BAD&lt;/sub&gt; promoter (Lee, 1980; Schleif, 1992).</td>
</tr>
</tbody>
</table>
pBAD202/D/lacZ (7,520 bp) is a control vector that contains the gene for \( \beta \)-galactosidase. The lacZ gene was amplified and directionally TOPO\textsuperscript{®} Cloned into pBAD202/D-TOPO\textsuperscript{®} in frame with HP-thioredoxin and the C-terminal peptide containing the V5 epitope and a polyhistidine (6×His) tag. The size of the \( \beta \)-galactosidase fusion protein is approximately 133 kDa.

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

Regulation of the PBAD Promoter

The araBAD promoter used in the pBAD/D-TOPO® vectors is both positively and negatively regulated by the product of the araC 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 O₂ and I₁ half sites of the araBAD 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 O₂ site and bind the I₂ site which is adjacent to the I₁ 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 I₁ and I₂.

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.

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

Proofreading DNA Polymerases

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

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Cat. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum® Pfx DNA Polymerase</td>
<td>100 units</td>
<td>11708-013</td>
</tr>
<tr>
<td>AccuPrime™ Pfx DNA Polymerase</td>
<td>200 reactions</td>
<td>12344-024</td>
</tr>
<tr>
<td>Pfx50™ DNA Polymerase</td>
<td>100 reactions</td>
<td>12355-012</td>
</tr>
</tbody>
</table>

Electrocompetent Cells

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

<table>
<thead>
<tr>
<th>Kit</th>
<th>Reactions</th>
<th>Cat. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Shot® TOP10 Electrocomp™ E. coli</td>
<td>10</td>
<td>C4040-50</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>C4040-52</td>
</tr>
<tr>
<td>TOP10 Electrocomp™ Kits</td>
<td>20</td>
<td>C664-55</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>C664-11</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>C664-24</td>
</tr>
</tbody>
</table>

Continued on next page
Accessory Products, Continued

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.

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

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.

<table>
<thead>
<tr>
<th>Product</th>
<th>Amount</th>
<th>Cat. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProBond™ Purification System</td>
<td>6 purifications</td>
<td>K850-01</td>
</tr>
<tr>
<td>ProBond™ Nickel-Chelating Resin</td>
<td>50 mL</td>
<td>R801-01</td>
</tr>
<tr>
<td></td>
<td>150 mL</td>
<td>R801-15</td>
</tr>
<tr>
<td>Ni-NTA Purification System</td>
<td>6 purifications</td>
<td>K950-01</td>
</tr>
<tr>
<td>Ni-NTA Agarose</td>
<td>10 mL</td>
<td>R901-01</td>
</tr>
<tr>
<td></td>
<td>25 mL</td>
<td>R901-15</td>
</tr>
<tr>
<td>Purification Columns</td>
<td>50 columns</td>
<td>R640-50</td>
</tr>
<tr>
<td>(10 mL polypropylene columns)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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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Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_support@invitrogen.com

Japanese Headquarters:
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Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:
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Paisley PA4 9RF, UK
Tel: 44 (0) 141 814 6100
Tech Fax: 44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

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.

Limited Warranty

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Introduction

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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.
References


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References, Continued


