Invitrogen™
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Instruction Manual

# pBAD TOPO® TA Expression Kit

## Five-minute cloning of *Taq* polymerase-amplified PCR products for regulated expression in *E. coli*

**Catalog nos. K4300-01, K4300-40**

**Version N**
13 April 2004
*25-0196*

# Table of Contents

# Kit Contents and Storage

## Types of Kits

This manual is supplied with the following kits.

| Kit | Quantity | Catalog no. |
|---|---|---|
| pBAD TOPO® TA Expression Kit | 20 reactions | K4300-01 |
| | 40 reactions | K4300-40 |

## Shipping and Storage

The pBAD TOPO® TA Expression Kit is shipped on dry ice. Each kit contains pBAD TOPO TA Cloning® reagents (Box 1), One Shot® TOP10 Chemically Competent *E. coli* (Box 2), and a small bag with an LMG194 stab.

**Store Box 1 at -20°C and Box 2 at -80°C. Store the LMG194 stab at room temperature.**

## TOPO® TA Cloning Reagents

pBAD TOPO TA Cloning® reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase.** Store Box 1 at -20°C.

| Item | Concentration | Amount |
|---|---|---|
| pBAD-TOPO® vector | 10 ng/µl plasmid DNA in:<br>50% glycerol<br>50 mM Tris-HCl, pH 7.4 (at 25°C)<br>1 mM EDTA<br>1 mM DTT<br>0.1% Triton X-100<br>100 µg/ml BSA<br>phenol red | 25 µl |
| 10X PCR Buffer | 100 mM Tris-HCl, pH 8.3 (at 42°C)<br>500 mM KCl<br>25 mM $MgCl_2$<br>0.01% gelatin | 100 µl |
| dNTP Mix<br>(50 mM dNTPs) | 12.5 mM dATP<br>12.5 mM dCTP<br>12.5 mM dGTP<br>12.5 mM dTTP<br>neutralized at pH 8.0 in water | 10 µl |
| 20% L-Arabinose | 20% in sterile water | 1 ml |
| pBAD Forward Sequencing Primer | 0.1 µg/µl in TE Buffer | 20 µl |
| pBAD Reverse Sequencing Primer | 0.1 µg/µl in TE Buffer | 20 µl |

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# Kit Contents and Storage, continued

### pBAD-TOPO TA Cloning® Reagents, continued

| Item | Concentration | Amount |
|---|---|---|
| Salt Solution | 1.2 M NaCl<br>0.06 M $MgCl_2$ | 50 µl |
| Control PCR Primers | 0.1 µg/µl in TE Buffer | 10 µl |
| Control PCR Template | 0.05 µg/µl in TE Buffer | 10 µl |
| Sterile Water | -- | 1 ml |
| Expression Control Plasmid<br>(pBAD-TOPO®/*lac*Z/V5-His) | 10 ng/µl | 10 µl |

### Sequences of pBAD Primers

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

| Primer | Sequence | pMoles Supplied |
|---|---|---|
| pBAD Forward | 5´-ATGCCATAGCATTTTTATCC-3´ | 350 |
| pBAD Reverse | 5´-GATTTAATCTGTATCAGG-3´ | 363 |

### One Shot® Reagents

The table below describes the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit. **Store at -80°C.**

| Item | Composition | Amount |
|---|---|---|
| S.O.C. Medium<br>(may be stored at room temperature or +4°C) | 2% Tryptone<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>20 mM glucose | 6 ml |
| TOP10 cells | -- | 21 x 50 µl |
| pUC19 Control DNA | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0 | 50 µl |

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# Kit Contents and Storage, continued

**Genotype of TOP10**

Use this strain for general cloning of blunt-end PCR products into the pBAD-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**

**Genotype:** 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*BAD*C*. It is also streptomycin and tetracycline resistant.

# Accessory Products

## Additional Products

Many of the reagents supplied with the pBAD-TOPO® TA Expression Kit and other reagents suitable for use with the kit are available separately from Invitrogen. Ordering information for these reagents is provided below.

| Product | Amount | Catalog no. |
|---|---|---|
| Platinum® *Taq* DNA Polymerase | 100 reactions | 10966-018 |
| | 250 reactions | 10966-026 |
| | 500 reactions | 10966-034 |
| *Taq* DNA Polymerase, Recombinant | 100 units | 10342-053 |
| | 500 units | 10342-020 |
| One Shot® TOP10 Chemically Competent Cells | 10 reactions | C4040-10 |
| | 20 reactions | C4040-03 |
| One Shot® TOP10 Electrocompetent Cells | 10 reactions | C4040-50 |
| PureLink™ HQ Mini Plasmid Purification Kit | 100 reactions | K2100-01 |
| EKMax™ Enterokinase | 250 units | E180-01 |
| Ampicillin | 20 ml | 11593-019 |
| 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 |

## Detection of Recombinant Proteins

Expression of your recombinant protein can be detected using 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.

| Epitope | Antibody | Catalog No. |
|---|---|---|
| Anti-V5 Antibody | Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991).<br>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 (6xHis) tag, requires the free carboxyl group for detection (Lindner *et al.*, 1997).<br>HHHHHH-COOH | R930-25 |
| Anti-His(C-term)-HRP Antibody | | R931-25 |
| Anti-His(C-term)-AP Antibody | | R932-25 |

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# Accessory Products, continued

## Purification of Recombinant Proteins

If your gene of interest in is frame with the C-terminal polyhistidine (6xHis) 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 | Catalog 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<br>(10 ml polypropylene columns) | 50 | R640-50 |

# Methods

## Overview

### Introduction

pBAD TOPO® TA Expression Kit provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for regulated expression in *E. coli*. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Expression in *E. coli* is driven by the *ara*BAD promoter ($P_{BAD}$). The AraC gene product encoded on the pBAD-TOPO® plasmid positively regulates this promoter.

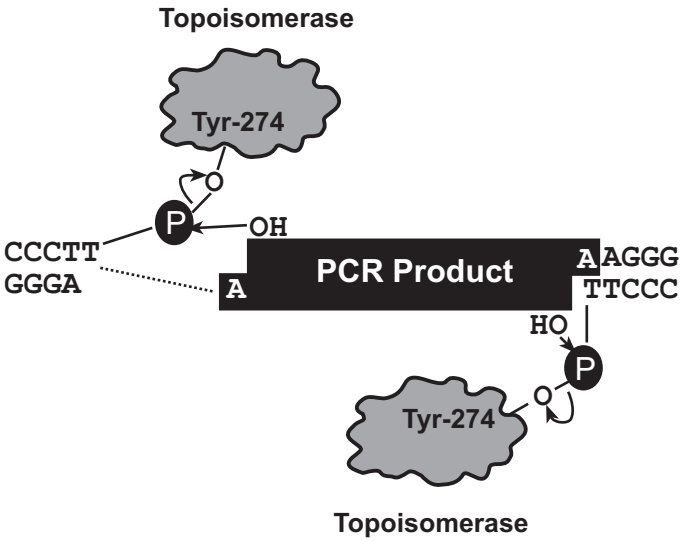
### TOPO® Cloning

The PCR expression vector (pBAD-TOPO®) is supplied linearized with:

- Single 3´-thymidine (T) overhangs for TA Cloning®
- Topoisomerase (bound to the vector)

*Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

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).

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# Overview, continued

## Regulation of Expression by L-Arabinose

In the presence of L-arabinose, expression from $P_{BAD}$ is turned on while the absence of L-arabinose produces very low levels of transcription from $P_{BAD}$ (Lee, 1980; Lee *et al.*, 1987). Uninduced levels are repressed even further by growth in the presence of glucose. Glucose reduces the levels of 3´, 5´-cyclic AMP, thus lowering expression from the catabolite-repressed $P_{BAD}$ promoter (Miyada *et al.*, 1984). By varying the concentration of L-arabinose, protein expression levels can be optimized to ensure maximum expression of soluble 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.

## Experimental Outline

The table below describes the general steps required to TOPO® Clone and express your gene of interest.

| Step | Action | Pages |
|---|---|---|
| 1 | Design PCR primers to clone your PCR product into the pBAD-TOPO® vector. | 3-4 |
| 2 | Produce your PCR product. | 5-6 |
| 3 | TOPO® Clone your PCR product into pBAD-TOPO®. | 7-8 |
| 4 | Transform the TOPO® Cloning reaction into One Shot® TOP10 *E. coli*. | 9-11 |
| 5 | Analyze transformants for the presence and orientation of the insert by restriction digestion, PCR, or sequencing. | 12-13 |
| 6 | Select positive transformants and induce expression with arabinose. | 15-17 |
| 7 | Purify your recombinant protein, if desired. | 20 |

# Designing PCR Primers

## Introduction

Before you may use the pBAD-TOPO® TA Expression Kit, you must first design PCR primers and produce your PCR product. Guidelines are provided in this section to help you design PCR primers.

## Factors to Consider

pBAD-TOPO® is designed with the following features to facilitate expression:

- The initiation ATG is correctly spaced from the optimized ribosome binding site to ensure optimum translation.
- A short N-terminal translation leader is provided which may improve expression of heterologous proteins in *E. coli.*

  **Note**: This N-terminal leader can be removed after protein purification using enterokinase (*i.e.* EKMax™, see page vii for ordering information).

**Important**

When synthesizing PCR primers, **do not** add 5′ phosphates to the primers as this will prevent the synthesized PCR product from ligating into the pBAD-TOPO® vector.

## Primer Design

Suggestions for primer design are provided in the table below. Remember that your PCR product will have 3′ adenine overhangs.

| If you wish to... | Then... |
|---|---|
| include the V5 epitope and polyhistidine region | design the reverse PCR primer to remove the native stop codon in the gene of interest and preserve the reading frame through the C-terminal tag. |
| **not** include the V5 epitope 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. |
| clone in frame with the N-terminal leader sequence | design the forward PCR primer to preserve the reading frame from the N-terminal leader peptide through your protein of interest. |
| remove the N-terminal leader (for expression of native protein) | design the forward PCR primer to include a unique *Nco* I site which contains the first ATG of the protein.<br>Example: 5′-ACC ATG G<br>Digest the vector *Nco* I after cloning and religate. Make sure there are no internal *Nco* I sites in your PCR product.<br>**OR**<br>design the forward PCR primer to include an in-frame stop codon and a translation reinitiation sequence consisting of a ribosome binding site and the first ATG of the protein spaced 7-14 bases apart.<br>Example: 5′- GAG GAA TAA TAA ATG |

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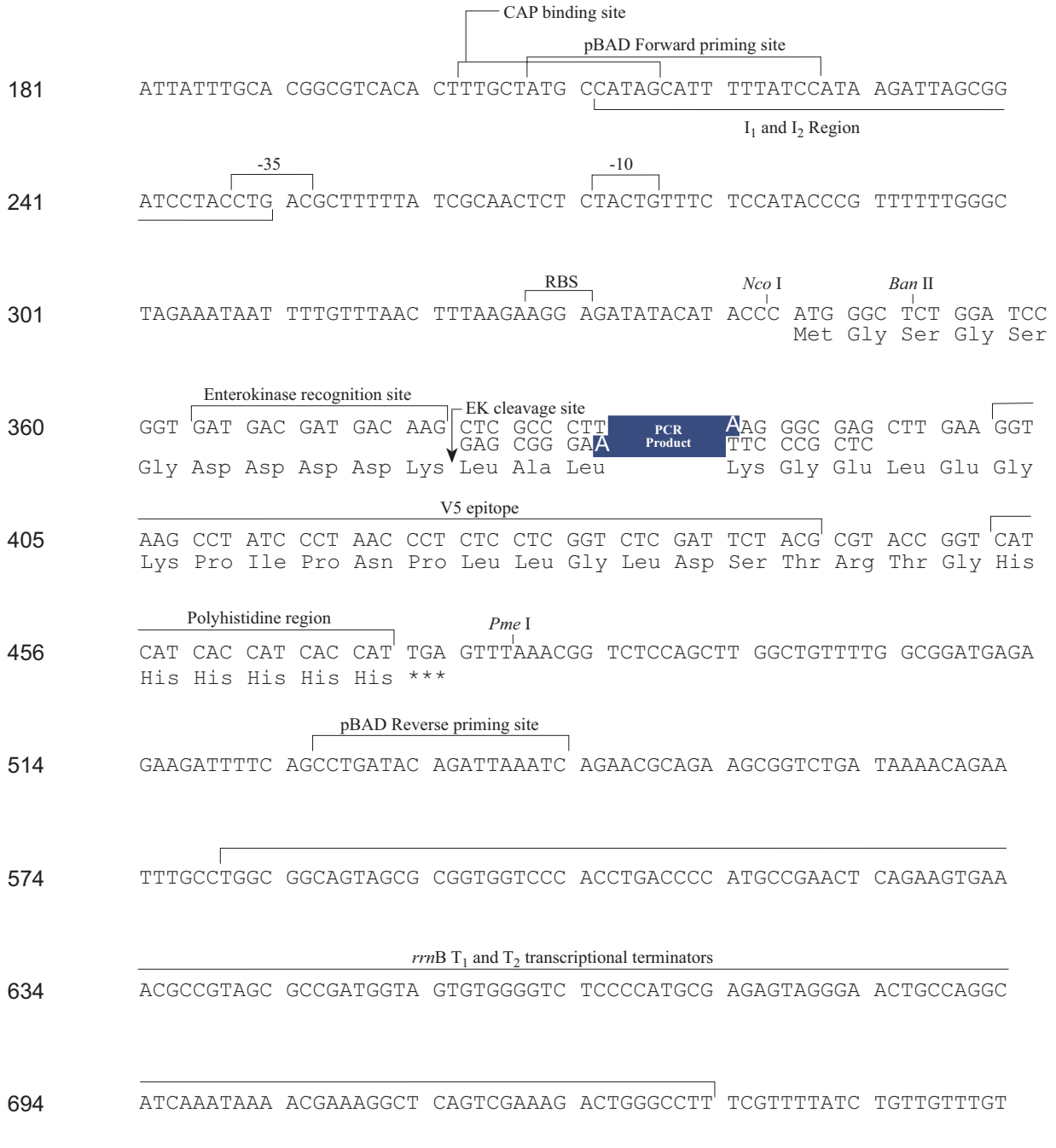
# Designing PCR Primers, continued

## TOPO® Cloning Site

Use the diagram below to help you design PCR primers to clone your PCR product into pBAD-TOPO®. Restriction sites are labeled to indicate the actual cleavage site.



# Producing PCR Products

## Introduction

Once you have synthesized appropriate PCR primers, you may use the primers and a suitable DNA polymerase to produce your PCR product. **Remember that your PCR product must have single 3′ A-overhangs.**

## Materials Needed

You should have the following materials on hand before beginning. **Note:** dNTPs (adjusted to pH 8) are provided in the kit.

- *Taq* polymerase

  **Note:** For improved specificity and higher yields, we recommend using Platinum® *Taq* DNA Polymerase available from Invitrogen (see page vii for ordering information) to generate your PCR product.
- Thermocycler
- DNA template and primers to produce your PCR product

## Polymerase Mixtures

You may use a polymerase mixture containing *Taq* polymerase and a proofreading polymerase to produce your PCR product; however, the mixture must contain a ratio of *Taq* polymerase:proofreading polymerase in excess of 10:1 to ensure the presence of 3′ A-overhangs on the PCR product.

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you may add 3′ A-overhangs to your PCR product using the method on page 29.

## Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full-length and 3′ adenylated.

| | |
|---|---|
| DNA Template | 10-100 ng |
| 10X PCR Buffer | 5 µl |
| dNTP Mix (50 mM) | 0.5 µl |
| PCR primers (100-200 ng each) | 1 µM each |
| Sterile water | add to a final volume of 49 µl |
| *Taq* Polymerase (1 U/µl) | 1 µl |
| Total volume | 50 µl |

2. Use agarose gel electrophoresis to verify the quality of your PCR product. You should see a single, discrete band of the correct size. If you do not see a single band, refer to the **Note** on the next page.

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# Producing PCR Products, continued

**Note**

If you do not obtain a single, discrete band from your PCR, try the following:

- Optimize your PCR to eliminate multiple bands and smearing (Innis *et al.,* 1990). The PCR Optimizer™ Kit available from Invitrogen (Catalog no. K1220-01) incorporates many of the recommendations found in this reference. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 34).
- Gel-purify your fragment using one of the methods on pages 27-28. Take special care to avoid sources of nuclease contamination.

# Setting Up the TOPO® Cloning Reaction

## Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pBAD-TOPO® vector and transform the recombinant vector into One Shot® TOP10 *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® TOP10 Competent Cells** (pages 9-11) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 21-23 in parallel with your samples.

**Note**

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction can increase the number of transformants 2- to 3-fold. In addition, incubating the reaction mixture for greater than 5 minutes in the presence of salt can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Including salt in the TOPO® Cloning reaction allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules, leading to higher transformation efficiencies.

## Using Salt Solution in the TOPO® Cloning Reaction

You will 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 vii 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 on the next page.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** 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 on the next page.

## Materials Needed

You should have the following materials on hand before beginning:

- Your PCR product (freshly prepared)
- pBAD-TOPO® vector (supplied with the kit, Box 1; keep at -20°C until use)
- Salt Solution (supplied with the kit, Box 1) or Dilute Salt Solution as appropriate
- Sterile water (supplied with the kit, Box 1)

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# Setting Up the TOPO® Cloning Reaction, continued

## Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction using the reagents in the order shown, and depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.*

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

| Reagent* | 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. Salt solution and water can be stored 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 will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied 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 pBAD-TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page vii for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

## Materials Needed

You should have the following materials on hand before beginning:

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot® TOP10 chemically competent *E. coli* (supplied with the kit, Box 2)
- S.O.C. Medium (included with the kit, Box 2)
- pUC19 positive control (to verify transformation efficiency, if desired, Box 2)
- 42°C water bath (or electroporator with cuvettes, optional)
- 15 ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 100 µg/ml ampicillin (two for each transformation)
- 37°C shaking and non-shaking incubator

## Preparing for Transformation

For each transformation, you will need one vial of One Shot® 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 100 µg/ml ampicillin at 37°C for 30 minutes.
- Thaw **on ice** one vial of One Shot® TOP10 cells for each transformation.

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# Transforming One Shot® TOP10 Competent Cells, continued

## One Shot® TOP10 Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 8 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

   **Note:** If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).

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.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. 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 should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 12).

## One Shot® Electroporation Protocol

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

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 8 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   **Note:** If you have problems with arcing, see the next page.

3. Immediately add 250 µl of room temperature S.O.C. Medium.
4. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake at 37°C for 1 hour.
5. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. 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 should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 12).

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# Transforming One Shot® TOP10 Competent Cells, continued


To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 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 10 colonies and culture them overnight in LB medium containing 100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
3. Analyze the plasmids by restriction analysis or PCR to confirm the presence and correct orientation of the insert.

## Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation and is in frame with the C-terminal V5 epitope and 6xHis tag, if desired. The pBAD Forward and pBAD Reverse sequencing primers are included in the kit to help you sequence your insert (see the diagram on page 4 for the location of the priming sites).

## Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the pBAD Forward and pBAD Reverse sequencing primers and a primer that hybridizes within your insert. You will have to determine the amplification conditions. 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 suitable.

**Materials Needed**

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

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. Visualize by agarose gel electrophoresis.

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# Analyzing Transformants, continued

## Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on an LB plate containing 100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 µg/ml ampicillin.
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.

# Optimizing the TOPO® Cloning Reaction

## Introduction

Use the information below to help you optimize the TOPO® Cloning reaction for your particular needs.

## Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high cloning efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

## More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

## Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

# Expressing the PCR Product

## Introduction

Since each recombinant protein has different characteristics that may affect optimum expression, it is helpful to vary the L-arabinose concentration and/or run a time course of expression to determine the best conditions for optimal expression of your particular protein.

## Use of 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. **Once you have determined that you have the correct construct, transform it into LMG194 prior to performing expression experiments.**

## Plasmid Preparation

You may prepare plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (see page vii for ordering information) for isolation of pure plasmid DNA. Note that since 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

pBAD-TOPO®/*lacZ*/V5-His is provided as a positive control for expression. This vector allows expression of a C-terminally tagged β-galactosidase fusion protein that may be detected by Western blot or functional assay. Transform 10 ng of the control plasmid into One Shot® TOP10 cells using the procedure on page 10.

## Basic Strategy

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

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

**Note**: If your protein is insoluble, remember to analyze the supernatant and the pellet of lysed cells for expression of soluble protein (page 18).

## Materials Needed

You should have the following materials on hand before beginning:

- SOB or LB containing 100 µg/ml ampicillin
- 37°C shaking incubator
- 20% L-arabinose (provided). Additional L-arabinose is available from Sigma (Catalog no. A3256).

*continued on next page*

# Expressing the PCR Product, continued

## Pilot Expression

For best results, we recommend including the pBAD-TOPO®/*lacZ*/V5-His transformants 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 100 µg/ml ampicillin with a single recombinant *E. coli* colony.

   **Note:** If you are using LMG194 as a host, use RM medium containing glucose and 100 µg/ml ampicillin at all steps (see page 25 for a recipe).

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 100 µg/ml ampicillin.
4. Inoculate each tube with 0.1 ml of the overnight culture.
5. Grow the cultures at 37°C with vigorous shaking to an $OD_{600}$ = ~0.5 (the cells should be in mid-log phase).
6. While the cells are growing, prepare four 10-fold serial dilutions of 20% L-arabinose with sterile water 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**, page 18.

*continued on next page*

# Expressing the PCR Product, continued

### Expressing Toxic Proteins

To ensure low levels of expression, you may find it useful to utilize glucose 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.
2. Follow the Pilot Expression protocol (see previous page) substituting RM medium containing glucose (see page 25 for recipe) to grow the cells.
3. Be sure to monitor the $OD_{600}$ as the cells will grow more slowly in RM medium.
4. Induce with various concentrations of arabinose as described in the Pilot Expression protocol.
5. Monitor $OD_{600}$ over time to be sure cells are growing.

# Analyzing Samples

## Materials Needed

You should have the following materials on hand before beginning:

- Reagents and apparatus for SDS-PAGE gel
- 1X and 2X SDS-PAGE sample buffer (see page 26 for recipes)
- Boiling water bath
- Lysis Buffer (see page 26 for recipe)
- Liquid nitrogen, optional

## Preparing Samples

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

1. When all the samples have been collected from the pilot expression, 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 26 for recipe).
2. Freeze sample in dry ice or liquid nitrogen and then thaw at 42°C. Repeat 2 to 3 times.

   **Note:** To facilitate lysis, you may need to add lysozyme 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 our Web site (www.invitrogen.com) or contact Technical Service (page 34).

*continued on next page*

# Analyzing Samples, continued

## Analyzing Samples

To determine the success of your expression experiment, you may want to 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).
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 should be approximately 120 kDa when induced with .02% arabinose.
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 vii for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5, or C-terminal 6xHis 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 our Web site (www.invitrogen.com) or contact Technical Service (page 34).

## Assay for β-galactosidase Activity

If you use the expression control plasmid, you may assay for β-galactosidase expression by Western blot analysis or activity assay using cell-free lysates (Miller, 1972). Invitrogen offers the β-Gal Antiserum, the β-Gal Assay Kit, and the β-Gal Staining Kit (see page vii for ordering information) for fast and easy detection of β-galactosidase expression.

**Note**

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

*continued on next page*

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# Analyzing Samples, continued

## Optimizing Expression

Once you have detected expression of your protein of interest, you may wish to perform some experiments to further optimize expression. Use the **Pilot Expression** protocol (page 16) but vary the arabinose concentration over a smaller range. For example, if you obtained the best expression at 0.002%, try 0.0004%, 0.0008%, 0.001%, 0.004%, and 0.008%.

Also you may perform a time course of induction 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, you may wish to analyze the supernatant and pellet of lysed cells when you vary the arabinose concentration (see **Preparing Samples for Soluble/Insoluble Protein**, page 18).

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

## Purifying Recombinant Fusion Proteins

The presence of the C-terminal polyhistidine (6xHis) tag in your recombinant fusion protein allows use of a metal-chelating resin such as ProBond™ to purify your fusion protein. The ProBond™ Purification System and bulk ProBond™ resin are available from Invitrogen (see page viii for ordering information). Refer to the ProBond™ Purification System manual for protocols to purify your fusion protein. Invitrogen also offers Ni-NTA Agarose (Catalog no. R901-01) for purification of proteins containing a polyhistidine (6xHis) tag**.** Other metal-chelating resins and purification methods are suitable.

## Removing the N-terminal Leader

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 three vector-encoded amino acids remaining at the N-terminus of the protein (see page 4).

A recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) is available from Invitrogen (see page vii for ordering information). To remove EKMax™ from the digest, EK-Away™ (Catalog no. R180-01) is also available.

# Appendix

## 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 containing the *lac* promoter and the LacZ fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing antibiotic and X-gal.

### Before Starting

Be sure to prepare the following reagents before performing the control reaction:

- 40 mg/ml X-gal in dimethylformamide (see page vii for ordering information)
- LB plates containing 100 µg/ml ampicillin and X-gal

### Producing Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter and LacZ , set up the following 50 µl PCR:

| | |
|---|---|
| Control DNA Template (50 ng) | 1 µl |
| 10X PCR Buffer | 5 µl |
| 50 mM dNTPs | 0.5 µl |
| Control PCR Primers (0.1 µg/µl) | 2 µl |
| Sterile Water | 40.5 µl |
| *Taq* Polymerase (1 unit/µl) | 1 µl |
| Total Volume | 50 µl |

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

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

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. 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-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 or Dilute Salt Solution | 1 µl | 1 µl |
| Control PCR Product | -- | 1 µl |
| pBAD-TOPO® vector | 1 µl | 1 µl |

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of TOP10 One Shot® cells using the protocol on page 10.
4. Spread 10-50 µl of each transformation mix onto LB plates containing 100 µg/ml ampicillin and X-Gal. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of S.O.C. to allow even spreading.
5. Incubate overnight at 37°C.

## What You Should See

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 90% of these will be blue and contain the 500 bp insert when analyzed by digestion with *Nco* I and *Pme* I.

## Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 10. 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 x $10^9$ cfu/µg DNA.

*continued on next page*

### Factors Affecting Cloning Efficiency

Note that lower cloning efficiencies will result from the following variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 90% (or more) cloning efficiency.

| Variable | Solution |
|---|---|
| Low efficiency of directional cloning | Forward primer should contain CACC at the 5′ end. |
| | Reverse primer is complementary to the overhang at the 5′ end. Redesign primer to avoid base pairing to the overhang. |
| pH>9 in PCR amplification reaction | Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8. |
| Incomplete extension during PCR | Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time. |
| Cloning large inserts (>1 kb) | Increase amount of insert or gel-purify as described on page 27. |
| Excess (or overly dilute) PCR product | Reduce (or concentrate) the amount of PCR product. |
| PCR cloning artifacts ("false positives") | TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product as described on page 27 or optimize your PCR. |

# Recipes

## LB (Luria-Bertani) Medium and Plates

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 harden, then invert 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 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 $MgCl_2$
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**

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

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

*continued on next page*

# Recipes, continued

## Lysis Buffer

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

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

   0.3 ml $KH_2PO_4$
   4.7 ml $K_2HPO_4$
   2.3 g NaCl
   0.75 g KCl
   10 ml glycerol
   0.5 ml Triton X-100
   68 mg imidazole
3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
4. Store at +4°C.

## 2X SDS-PAGE Sample Buffer

1. Combine the following reagents:

| | |
|---|---|
| 0.5 M Tris-HCl, pH 6.8 | 2.5 ml |
| Glycerol (100%) | 2.0 ml |
| β-mercaptoethanol | 0.4 ml |
| Bromophenol Blue | 0.02 g |
| SDS | 0.4 g |

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

## 1X SDS-PAGE Sample Buffer

1. Combine the following reagents:

| | |
|---|---|
| 0.5 M Tris-HCl, pH 6.8 | 1.25 ml |
| Glycerol (100%) | 1.0 ml |
| β-mercaptoethanol | 0.2 ml |
| Bromophenol Blue | 0.01 g |
| SDS | 0.2 g |

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

# Gel Purifying PCR Products

## Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish 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. Three simple protocols are provided below.

**Note**

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 5).

## Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   **Note**: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 8.

## Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (see page 8) Be sure to make the gel slice as small as possible for best results.

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# Gel Purifying PCR Products, continued

### Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

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. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 8.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® TOP10 cells using the protocol on page 10.

**Note**

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

# Addition of 3´ A-Overhangs Post-Amplification

## Introduction

Direct cloning of DNA amplified by proofreading polymerases into TOPO TA Cloning® vectors is often difficult because proofreading polymerases remove the 3′ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

## Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

## Procedure

This is just one method for adding 3′ adenines. Other protocols may be suitable.

1. After amplification with a proofreading polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer. A sufficient number of PCR products will retain the 3′ A-overhangs.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place on ice and use immediately in the TOPO® Cloning reaction.

**Note**: If you plan to store your sample overnight before proceeding with TOPO® Cloning, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR.

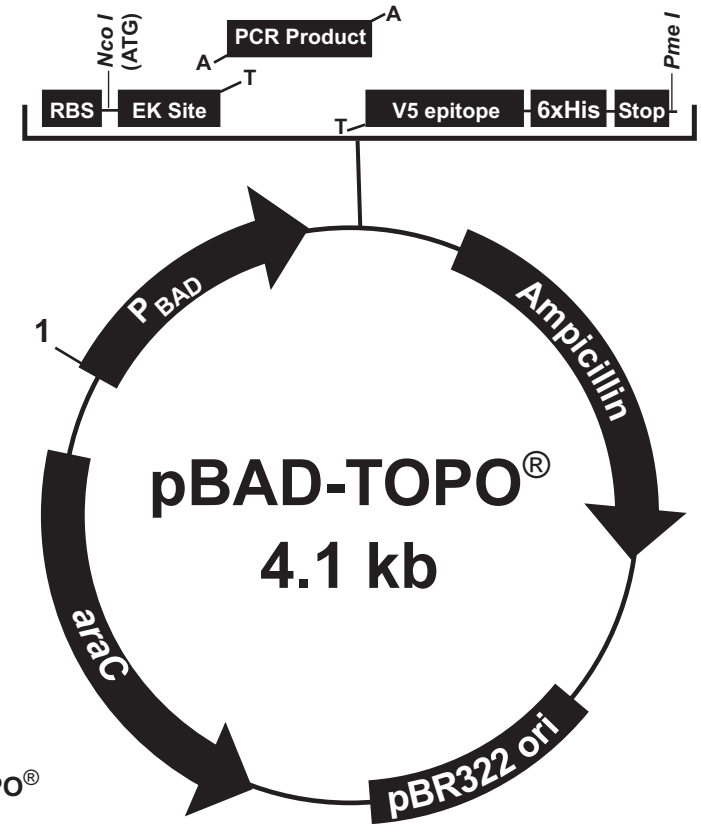
**Note**

You may also gel-purify your PCR product after amplification with a proofreading polymerase. After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase. Incubate the reaction for 10-15 minutes at 72°C and use in the TOPO® Cloning reaction.

# Map and Features of pBAD-TOPO®

## pBAD-TOPO® Map

The map below shows the features of pBAD-TOPO®. **The complete sequence of pBAD-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 34).**




**Comments for pBAD-TOPO®**
**4126 nucleotides**

Note: The vector is supplied linearized between bp 387 and bp 388. This is the TOPO® Cloning site.

Arabinose promoter and regulatory elements: bases 4-276
pBAD Forward priming site: bases 208-227
Ribosome binding site: bases 328-331
Initiation ATG codon: bases 345-347
Enterokinase recognition site: bases 363-377
TOPO® Cloning site: bases 387-388
V5 epitope: bases 402-443
Polyhistidine region: bases 453-470
pBAD Reverse priming site: bases 526-543
rrnB T1 and T2 transcription terminators: bases 576-733
Ampicillin resistance gene: bases 1013-1873
pBR322 origin: bases 2018-2691
AraC ORF: bases 4100-3222 (ORF on the opposite strand)

*continued on next page*

# Map and Features of pBAD-TOPO®, continued

## Features of pBAD-TOPO®

pBAD-TOPO® (4126 bp) contains the following elements. All features have been functionally tested. For more information on the regulation of gene expression by L-arabinose, see page 33.

| 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 $P_{BAD}$. |
| Optimized ribosome binding site | Increases efficiency of recombinant fusion protein expression. |
| Initiation ATG | Provides a translational initiation site for the fusion protein. |
| TOPO® Cloning site | Allows rapid cloning of your PCR product for expression. |
| C-terminal V5 epitope tag (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) | Allows detection of the fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991). |
| C-terminal polyhistidine (6xHis) region | Allows purification of the recombinant fusion protein on metal-chelating resins (*e.g.* ProBond™).<br>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. |
| Ampicillin resistance gene | Allows selection of the plasmid in *E. coli*. |
| pBR322 origin | Low copy replication and growth in *E. coli*. |
| *ara*C gene | Encodes the regulatory protein for tight regulation of the $P_{BAD}$ promoter (Lee, 1980; Schleif, 1992). |

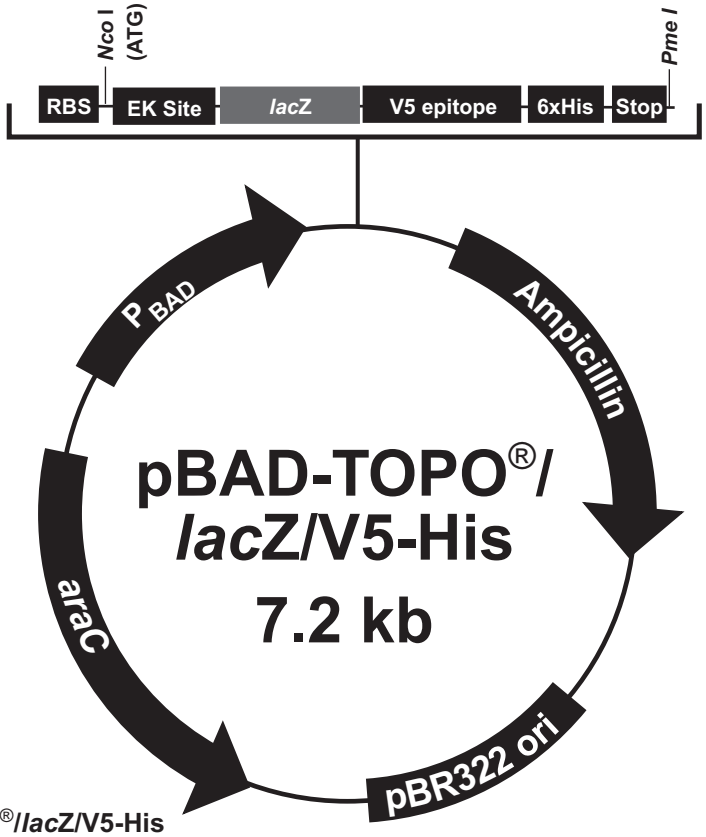
# Map of pBAD-TOPO®/*lacZ*/V5-His

## Description

pBAD-TOPO®/*lacZ*/V5-His is a 7183 bp control vector containing the gene for β-galactosidase fused to the C-terminal peptide. The vector expresses a 120 kDa protein which may be excised with *Nco* I and *Pme* I.

## Map of Control Vector

The figure below summarizes the features of the pBAD-TOPO®/*lacZ*/V5-His vector. **The complete nucleotide sequence for pBAD-TOPO®/*lacZ*/V5-His is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 34).**




**Comments for pBAD-TOPO®/*lacZ*/V5-His**
**7183 nucleotides**

Arabinose promoter and regulatory elements: bases 4-276
Ribosome binding site: bases 328-331
Initiation ATG codon: bases 345-347
Enterokinase recognition site: bases 363-377
*LacZ* ORF: bases 387-3443
V5 epitope: bases 3459-3500
Polyhistidine region: bases 3510-3527
*rrn*B T1 and T2 transcription terminators: bases 3633-3790
Ampicillin resistance gene: bases 4070-4930
pBR322 origin: bases 5075-5748
AraC ORF: bases 6279-7157 (complementary strand)

# Regulation by L-Arabinose

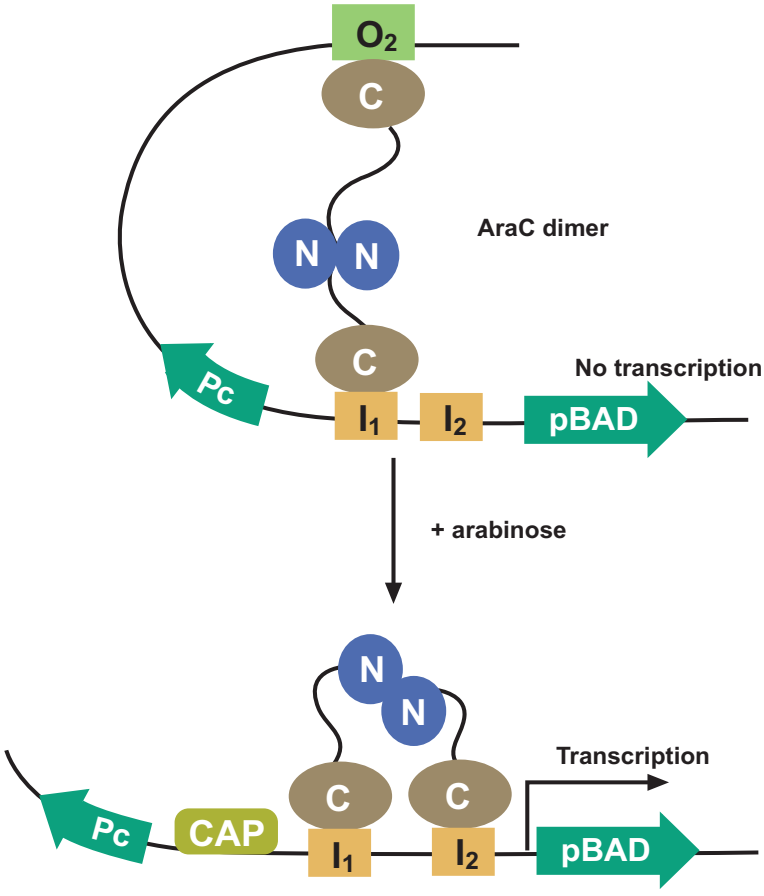
## Introduction

A brief description of the L-arabinose regulatory circuit is provided below.

## Regulation of the $P_{BAD}$ Promoter

The *ara*BAD promoter used in pBAD-TOPO® 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 L-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.

- L-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.

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# Product Qualification

| | |
|---|---|
| **Introduction** | The section describes the criteria used to qualify the components of the pBAD TOPO® TA Expression Kit. |
| **Vectors** | The parental supercoiled pBAD and pBAD-TOPO®/*lacZ*/V5-His are qualified by restriction digest prior to adaptation with topoisomerase. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel. |
| **TOPO® Cloning Efficiency** | After adaptation with topoisomerase I, pBAD-TOPO® is lot-qualified using the control reagents included in the kit. Under conditions described on pages 21-22, a 500 bp control PCR product is amplified, TOPO® Cloned into pBAD-TOPO®, and transformed into the One Shot® competent *E. coli* included with the kit.<br>Each lot of vector should yield greater than 90% cloning efficiency. |
| **Primers** | Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique. |
| **One Shot® TOP10 Competent *E. coli*** | One Shot® TOP10 competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1 x $10^9$ cfu/µg plasmid DNA.<br>In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and lack of phage contamination. |

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