invitrogen™

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

Revision date : 21 July 2009

Manual part no. 25-0196

MAN0000054

User Manual

Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Quantity	Cat. 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 4°C.

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: 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 phenol red	25 µL
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µL
dNTP Mix (50 mM dNTPs)	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP 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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pBAD-TOPO TA Cloning® Reagents, continued

Item	Concentration	Amount
Salt Solution	1.2 M NaCl 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 (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
TOP10 Cells	--	21 × 50 µL
S.O.C. Medium (may be stored at room temperature or 4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µL

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Genotype of TOP10

Use this strain for general cloning of 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*BADC. It is also streptomycin and tetracycline resistant.

Preparing LMG194 Glycerol Stocks

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

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

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

Introduction

Description of the System

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.

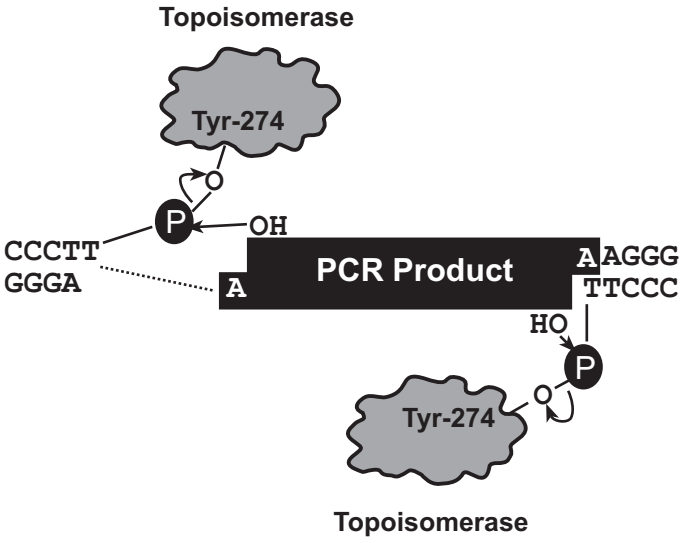
TOPO® Cloning

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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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 32 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

Methods

Designing PCR Primers

Introduction

Before using 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.

ATG Start Codon

pBAD-TOPO® is designed with the initiation ATG is correctly spaced from the optimized ribosome binding site to ensure optimum translation.

Important

When synthesizing PCR primers, **do not** add 5′ phosphates to the primers, because 5′ phosphates 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. Example: 5′-ACC ATG G… Digest the vector *Nco* I after cloning and religate. Make sure there are no internal *Nco* I sites in your PCR product. **OR** 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. Example: 5′- GAG GAA TAA TAA ATG…

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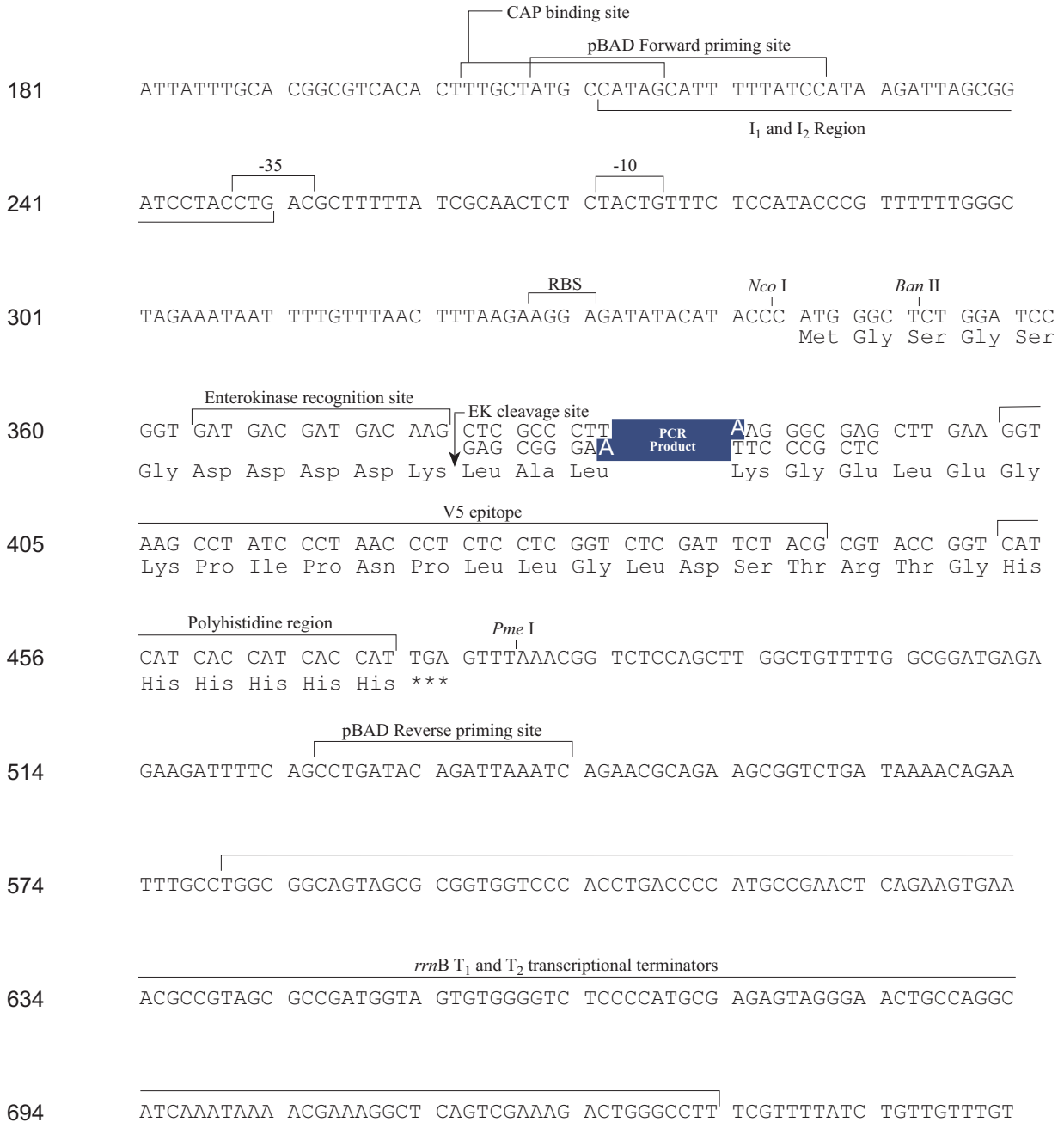
Designing PCR Primers, Continued

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

After you have synthesized appropriate PCR primers, 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

- *Taq* polymerase

  Note: For improved specificity and higher yields, we recommend using Platinum® *Taq* DNA Polymerase available from Invitrogen (see page 33 for ordering information) to generate your PCR product.
- Thermocycler
- DNA template and primers to produce your PCR product

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

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 25.

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.

Continued on next page

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 separately from Invitrogen, incorporates many of the recommendations found in this reference (see page 33 for ordering information).
- Gel-purify your fragment using one of the methods on pages 23–24. Take special care to avoid sources of nuclease contamination.

TOPO® Cloning Reaction

Introduction

After you have produced the desired PCR product, TOPO® Clone your product into the pBAD-TOPO® vector and transform the recombinant vector into One Shot® TOP10 *E. coli*. 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 26–28 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

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 33 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

- 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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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.* An Insert:vector molar ratio of 1:1 gives the optimal efficiency in TOPO® Cloning reaction.

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

Reagent[1]	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product[2]	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 of incubation yields 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

After you have performed the TOPO® Cloning reaction, 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 33 for ordering information). This section includes protocols to transform chemically competent or electrocompetent *E. coli*.

Materials Needed

- 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 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).

Continued on next page

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µL for 0.1 cm cuvettes or between 100 to 200 µL for 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 or PureLink™ HiPure Plasmid Miniprep kits (see page 33 for ordering information). Refer to www.invitrogen.com or contact Technical Support for more information on a large selection of plasmid purification columns.
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 6×His 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 also suitable.

Materials Needed

PCR SuperMix High Fidelity (see page 33 for ordering information)

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.

Continued on next page

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 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 glycerol stock at –80°C, and a stock of plasmid DNA at –20°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.

Using LMG194

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

Follow the guidelines below for using LMG194:

- Induce the pBAD promoter when cells are growing in LB or RM-Glucose.
- If you are growing your construct under maximal repression, i.e., with D-glucose in RM media, then you must spin down the culture and resuspend it in RM containing 0.2% glycerol and Arabinose (i.e., substitute glycerol for the glucose in the media recipe on page 22).

Plasmid Preparation

You may prepare plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification or PureLink™ HiPure Plasmid Miniprep kits for isolating pure plasmid DNA (see page 33 for ordering information). Refer to www.invitrogen.com or contact Technical Support for more information on a large selection of plasmid purification columns.

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

Positive Control

pBAD-TOPO®/*lac*Z/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 (preferred method) or functional assay. Transform 10 ng of the control plasmid into One Shot® TOP10 cells using the procedure on page 10.

Basic Strategy

We recommend the following strategy to determine the optimal expression level from your clones:

1. **Pilot Expression.** 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, try L-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 (page 18).

Continued on next page

Expressing the PCR Product, Continued

Materials Needed

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

Pilot Expression

For best results, we recommend including the pBAD-TOPO®/*lac*Z/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 for overnight growth (see page 22 for a recipe), and then substitute glycerol for glucose at Step 3 (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 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 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) using RM medium containing 0.2% glycerol to grow the cells (i.e., substitute glycerol for glucose in the media recipe on page 22).
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

- Reagents and apparatus for SDS-PAGE gel
- 1X and 2X SDS-PAGE sample buffer
- Boiling water bath
- Lysis Buffer (see page 22 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 22 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 www.invitrogen.com or contact Technical Support (page 35).

Continued on next page

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); this is the preferred method.
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 0.02% 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 34 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 detecting fusion proteins that contain a V5 or a 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 35).

Note

Expressing your recombinant fusion protein with the C-terminal tag increases 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

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 (6×His) 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 34 for ordering information). Refer to the ProBond™ Purification System manual for protocols to purify your fusion protein. Invitrogen also offers Ni-NTA Agarose (Cat. no. R901-01) for purification of proteins containing a polyhistidine (6×His) 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. To remove EKMax™ from the digest, you may use EK-Away™ Resin, also available from Invitrogen (see page 33 for ordering information).

Appendix

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

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.

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.

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 33 for ordering information).

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

Continued on next page

Low-Melt Agarose Method

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

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Use 4 µL of the melted agarose containing your PCR product in the TOPO® Cloning reaction (page 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 TOP10 One Shot® cells using the method on pages 9–11.

Note

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

Adding 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.

Materials Needed

- *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 are also 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 (see previous page for protocol). 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.

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 33 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. 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

3. 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

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

The vector + PCR insert reaction should yield hundreds of colonies. Greater than 90% of the colonies will be blue and contain the 500 bp insert when analyzed by *Nco* I and *Pme* I digestion.

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 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 ~1×10^9 cfu/µg DNA.

Continued on next page

Factors Affecting Cloning Efficiency

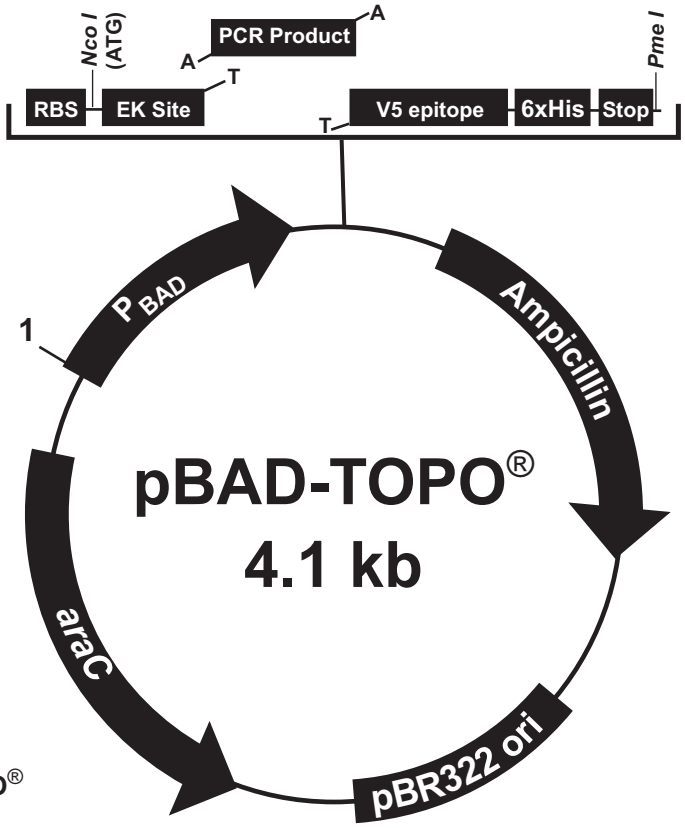
Note that lower cloning efficiencies result from the following variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 90% (or more) cloning efficiency.

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.
Insert:Vector molar ratio	Insert:Vector molar ratio of 1:1 will give the optimal efficiency in a TOPO® Cloning reaction.
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 23.
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 23 or optimize your PCR.

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 at www.invitrogen.com or by contacting Technical Support (page 35).**



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® (4,126 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 32.

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™). 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).

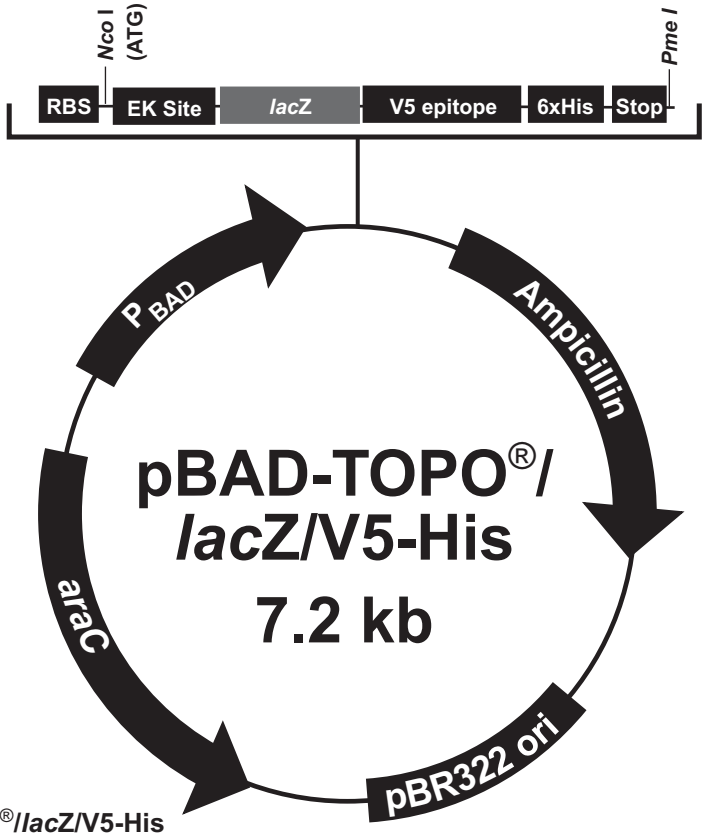
Map of pBAD-TOPO®/*lacZ*/V5-His

Description

pBAD-TOPO®/*lacZ*/V5-His is a 7,183 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 at www.invitrogen.com or by contacting Technical Support (page 35).**



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

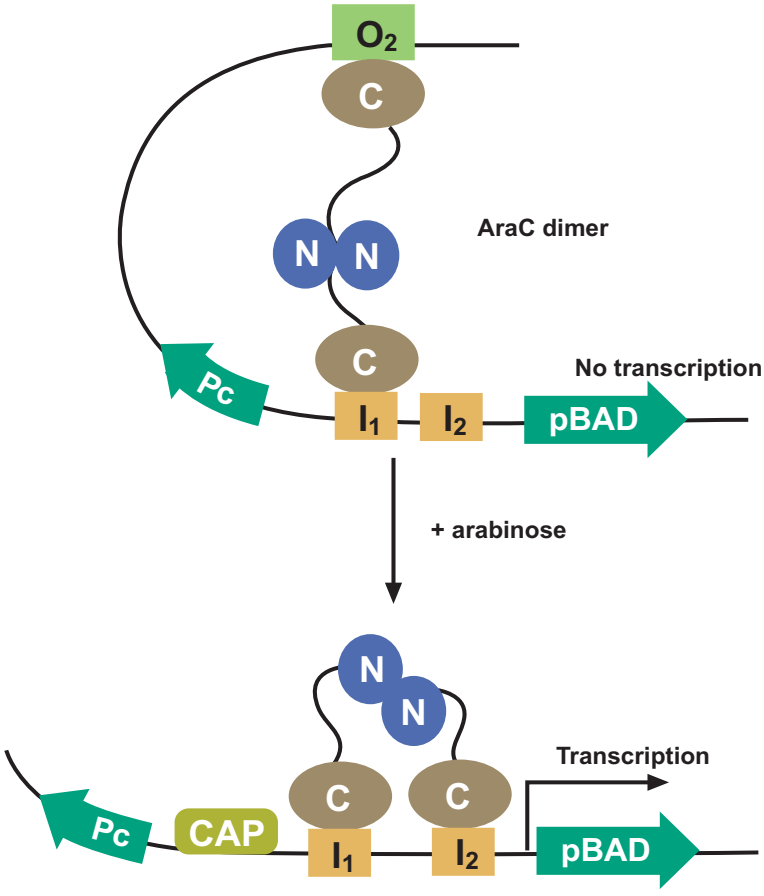
Introduction

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.

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. For details, visit www.invitrogen.com.

Product	Amount	Cat. 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
PCR Optimizer™ Kit	100 reactions	K1220-01
PCR SuperMix High Fidelity	100 reactions	10790-020
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink™ HiPure Plasmid Miniprep Kit	25 preps	K2100-02
	100 preps	K2100-03
EKMax™ Enterokinase	250 units	E180-01
EK-Away™ Resin	7.5 mL	R180-01
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
β-Gal Antiserum	50 µL	R901-25
β-Gal Assay Kit	100 reactions	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
X-gal	100 mg	15520-034

Continued on next page

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	Cat. no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991). GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His(C-term) Antibody	Detects the C-terminal polyhistidine (6×His) tag, requires the free carboxyl group for detection (Lindner *et al.*, 1997). HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25

Purification of Recombinant Proteins

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

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

Technical Support

Web Resources

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

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

Contact Us

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

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